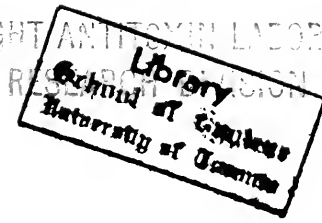






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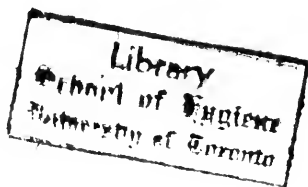


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# A COMPARISON OF THE ANTIGENIC PROPERTIES OF DIFFERENT STRAINS OF BACILLUS TYPHOSUS

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The use of bacterial preparations in the prophylactic and therapeutic management of many of the infections is gradually becoming more successful as the element of strain specificity among bacteria is observed and studied. Various immunologic and biochemic methods have been adapted and applied to the investigation of essential differences among families of bacteria which previously, and erroneously, had been regarded as definite entities, one strain being identical with all others.

Classificatory examination of numerous strains of pneumococci has eventuated in the discovery of definite groups which possess conspicuous antigenic individualities (24, 8); even among the members of one of these groups there have been found types which are differentiable by further refinement of serologic technic (1).

Significant antigenic dissimilarities among closely related streptococci have been determined by a number of investigators (32) by means of agglutination and alexin-fixation reactions. Pertussis bacilli have been found to be separable from influenza bacilli (26) and the heterogeneity of the influenza group (27) has been established by means of alexin-fixation, agglutination and agglutinin-absorption.

Immunologists have long been cognizant of the severally specific antigenic differences possessed by strains of gonococci (33), and recently the members of the allied species of meningo-

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cocci (25) have been found to be distributable into a few well-defined groups.

The serologic methods utilized in these researches have also been successfully applied in similar studies of the diphtheria (16), acid-fast (4) and colon-typhoid (17) groups of bacilli. Of these methods, alexin-fixation, where used, has been found to be the most delicate and the most specific, findings which are in accord with previous experience with this reaction in other fields.

The value of prophylactic antityphoid inoculation is now very generally admitted; of late there have come a number of hopeful reports of the use of various preparations of typhoid bacilli in the treatment of typhoid fever (9). There exists, however, a not inconsiderable number of instances in which inoculation has failed to protect (30, 34). These failures may, of course, be ascribed to absence or torpor of the individuals' reactivity, or to an extraordinarily large infecting dose, surely not negligible factors, although, with reference to the size of the dose, the mortality from laboratory infections with typhoid, in which the infecting dose is presumably large, is no higher than the average (14).

On the other hand, there are certain observational data which give warrant to the suspicion that the failures may be due to infection with a strain of typhoid bacilli which differs materially from the strain or strains used for immunizing. The fact that immunization of an animal with a Type I pneumococcus or with its antiserum confers no appreciable immunity to subsequent infection with a Type II organism is an explanatory, or better, a suggestive, analogy. A more germane illustration of the specificity of the protective forces which are aroused or prepared for mobilization by active immunization, is to be found in the reports of Castellani (2) and others (35), and in contemporary experience with the troops in Europe, which have abundantly demonstrated that *antityphoid* inoculation affords little or no protection against *paratyphoid* infections.

Moreover, the fluctuant local severity of typhoid epidemics from year to year, directly suggests a corresponding variation in the virulence of infecting strains. Strong (31), working with

cholera organisms, and Pfeiffer and Kolle (28) with *B. typhosus*, have demonstrated very considerable antigenic differences between virulent and relatively avirulent strains with regard to the protection conferred by active immunization.

Investigation of the diagnostic worth of the alexin-fixation reaction in typhoid fever has disclosed a fact which is more than suggestive. In order to secure the maximum number of positive reactions it is necessary to use a highly polyvalent antigen. Garbat (3) cites an illustrative case in which the serum gave strong fixation when the patient's own strain was used as antigen, but which gave negative reactions when tested with seven other strains of *B. typhosus*. Similar instances have been encountered in the experience of the writers.

These analogies and incidental observations, while not determinative in character, were considered sufficiently consonant to justify a detailed study of a number of strains of *B. typhosus*, with attention focused particularly upon the possible existence of antigenic differences which might be susceptible of demonstration by serologic methods. The revelation of such individualities would be probably of minor significance from an etiologic viewpoint, but for specific therapy and prophylaxis it would be of primary importance. The larger part, by far, of typhoid vaccine now used is prepared from a single strain and even the few attempts which have been made to provide for all possibilities by using a polyvalent vaccine are quite empirical; the only criterion of differentiation of strains has been difference in origin, with the sequential probability that many important strains have been omitted and that there has been unnecessary duplication of others.

The method of cross-agglutination seemed unsuited for use in this proposed study. It is only sometimes true that a serum immune to one typhoid strain agglutinates this strain in higher dilution than it does other typhoid strains. This irregularity is due to the various inherent agglutinabilities possessed by different strains, and obviously precludes the possibility of obtaining classificatory data by means of cross-agglutination. In agglutinin-absorption experiments, however, this agglutinability

factor can be controlled so that it does not seriously obscure interpretation of results. The alexin-fixation reaction was the method of choice because it has unquestionably established its differentiating value in similar investigations of other groups of bacteria. The precipitin reaction was not tried because it is not so capable of detecting finer characteristics as is the alexin-fixation reaction. Although our knowledge of the applicability of the conglutination reaction is very meager, the specific findings which it has afforded in studies of the dysentery (11, 20) group commend it to our earnest consideration. It failed in streptococcus differentiation (32), however, and little or nothing is known concerning the possible disturbing effect of individual agglutinabilities of members of the same species upon the evaluation of results. But these are not discouraging circumstances and the reaction would have been utilized in these studies had not the alexin-fixation and the agglutinin-absorption experiments consumed so much time.

#### ALEXIN-FIXATION EXPERIMENTS

##### *Materials and technic*

*Sources of strains.* Of the fifty-three strains of *B. typhosus* that have been studied, twenty-one are laboratory strains isolated from two to fifteen or more years ago, and sub-cultured on agar at one- to two-month intervals. The histories of those strains upon which the most work has been done are here appended.<sup>2</sup> The remaining thirty-two have been isolated and

<sup>2</sup> 1. Isolated June, 1912, from tibial abscess of patient that had typhoid fever in January, 1912.

3. Isolated by blood culture from girl in University of California Infirmary, November, 1912. Second subplant from blood culture.

4. Isolated October, 1913, by blood culture from patient that had been previously immunized.

5. "Dorset" strain, obtained from J. G. Fitzgerald, November, 1912.

7. Same history as No. 3, except that it was received from the California State Board of Health, October, 1913.

9. "Hopkins" strain, obtained from California State Board of Health, October, 1913.

13. "Rawlings" strain obtained from the Presidio, November, 1913. Originally from the Army Medical School.

authenticated by Gay and Chickering (9) during the past year in the course of studies upon the specific treatment of cases of typhoid fever occurring in Berkeley and its environs. All are motile, Gram-negative bacilli which ferment dextrose without gas formation, produce slight primary acidity in litmus-milk, do not ferment lactose, and are agglutinated to the titer limit by a known antityphoid serum. It is hardly necessary to state that such precautions as repeated examination of Gram-stained smears, plating, and confirmatory cultural and agglutinative tests have been carried out on each lot of antigen prepared, and it is felt that the errors which would arise from the use of mixed cultures as antigens and immunizing materials have been avoided. All cultures were plated and a single colony was used as the starting point of antigen preparation.

*Antigens.* The problem of preparing and preserving antigens of a sufficiently long range and delicate specificity to be used in cross-fixation tests involving scores of strains is of no mean magnitude. After a number of preliminary experiments the following method was adopted because of its comparative simplicity and the general usefulness of the final product.

Blake bottles, containing +0.5 beef-infusion agar, after being inoculated with fresh saline emulsion of a pure strain of *B. typhosus*, are incubated for four days. Subcultures and smears are then made for the confirmation of purity, 20 cc. of 0.1 per cent formaldehyde (0.25 per cent formalin) in 0.85 per cent sodium chlorid solution are added to each bottle, the growth rocked off and poured into large centrifuge tubes where it remains until sterile (23). Individual strains differ somewhat in regard to the time necessary for sterilization by this strength of formol. Three days, with occasional shaking of the tubes, represent the average. Then, after centrifugation at high speed, prolonged until the supernatant fluid is clear or but slightly turbid, the fluid is decanted, formolized salt solution is added to make up the original volume and the suspension is very thoroughly shaken and then filtered through sterile cotton wool in order to remove clumps of bacteria, bits of agar or other foreign material. If the growth is comparatively scanty, only enough

solution is added to make a suspension approximately of the same density as are those prepared from average growths; if it is very luxuriant, correspondingly more solution is used. The final suspension is milky-white, stable, not easily infected and it possesses the homogeneity and the freedom from spontaneous flocculation which are essential for agglutination tests.

These properly diluted suspensions of individual strains have been used as antigens in the alexin-fixation reaction, as reagents in agglutination tests (23) and as inocula for the production of immune sera in rabbits. They have been eminently satisfactory for all these purposes.

Unwashed suspensions probably would serve as well for agglutination tests and inoculations, but they are antigens of very short range<sup>3</sup> for use in alexin-fixation because of their strong auto-inhibitive (anticomplementary) property. This non-specific adsorption or inhibition of alexin, is due chiefly to material extracted from the culture medium. The formolized saline extract of the agar in an uninoculated Blake bottle is quite as anticomplementary as are many of the *unwashed* suspensions.

Washed suspensions have fixation indices; i.e., the anticomplementary dose divided by the antigenic unit obtained with homologous immune serum, ranging from 10 to 200, averaging about 40. Cross-fixation tests are more highly specific when the antigens have such long ranges. The presence of formol in these antigens has no effect upon hemolysis, fixation or agglutination except perhaps to render the last more sensitive.

Other preparations have been investigated as to their value as antigens for the fixation reaction. Among them are bouillon filtrates, bouillon cultures killed by heat; suspensions, extracts and resuspended sediments of alcohol-killed, dried, ground bacilli combined with various preservatives. None of the samples tried has shown any remarkable superiority over the washed suspensions, while many have been much more difficult to prepare and many have been inferior in uniformity and stability. The claim is not made that the formol-killed, washed

<sup>3</sup> An antigen is said to have a short range when there is small difference between its anticomplementary and its antigenic doses.

suspension is the ideal antigen. It is likely that an aqueous autolyzate of the *washed* suspension would serve as well and it would remove the objection to all suspensions—their turbidity. This cloudiness, which is apparent in the lower dilutions of antigen, interferes somewhat with the determination of clear-cut hemolysis when titrating for the anticomplementary dose. If, however, the tubes are not shaken during the last half-hour of incubation and are gently flipped when read, the tiny swirl of the sedimented corpuscles which then appears, affords a uniform and satisfactory standard of hemolysis determination in spite of the existing slight haziness of the tube contents.

*Immune sera.* Normal, well-grown rabbits were injected intravenously with approximately one-hundredth part of a formolized four-day growth in a Blake bottle. This dose remained constant and was given usually at intervals of four or five days unless the reaction was too severe. On both the fifth and sixth, or sixth and seventh, days after the fourth or fifth injection, from 25 to 50 cc. of blood were taken by cardiac puncture. In spite of repeated punctures, less than 10 per cent of the animals died of acute hemopericardium. When the need of more serum was anticipated, the animals were reinjected three times with their original strains and bled as above. In all, as much as 150 cc. of blood have been taken from time to time from a single rabbit. Excepting specimens to be used in agglutinin-absorptions, all sera have been heated for thirty minutes at 56°C., and have also been freshly inactivated just before fixation tests were carried out.

One serum with an agglutinin titer of 8000 gave no fixation; but most sera have averaged very high in agglutinins, 8000 to 64,000 and in fixation antibodies 0.004 to 0.00008 cc., with the technic to be described. These results are in keeping with those of Reiter and Silberstein (29), who found that, in suitable dosage, more agglutinins and tropins were produced in doves when the typhoid bacilli were formol-killed than when they were sterilized by heat, chloroform, ether, phenol, or ozone.

The method of rapid immunization (10, 12) in which injections are given on three successive days followed by bleeding a

week later, did not give sera of sufficiently high fixation-antibody content to be used in cross-titrations, although agglutinins were present in moderately high dilutions.

*The hemolytic system.* The rabbit-anti-sheep system has been used. Alexin has been preserved by salting; the pooled sera of six or more guinea pigs being mixed with an equal volume of 17 per cent sodium chlorid solution. A quantity sufficient for the day's test is isotonized by the addition of nine parts of distilled water and any further necessary dilution of this 5 per cent alexin is made with 0.85 per cent saline. Aside from the great convenience of this method it is deemed of unquestionable advantage to have one lot of alexin of practically uniform fixability and hemolytic potency with which a large number of strictly comparable cross-fixations may be made. The titer of this salted serum depreciates no more than 0.001 to 0.002 cc. in a month if sterility is maintained.

Alexin and immune hemolysin have been simultaneously standardized, sensitized cells being used, on each day that fixation tests have been made. The fundamental importance of a uniformly balanced hemolytic system in work of this nature is well recognized, and inasmuch as this particular method of standardization has not before been described, its details will be discussed at some length.

In titrating alexin with a fixed volume of cells sensitized with graded dilutions of hemolysin, it is found that there exists a distinct "hemolytic zone" within the limits of which the alexin titer—read at the end of an hour—is constant. Beyond the lower limit of this zone, which usually lies between 25 and 40 units of hemolysin, the titer of alexin becomes less. Beyond the upper limit, between 1.5 and 2 hemolysin units, the titer of alexin rises. By a hemolysin unit is meant the smallest amount of hemolysin which causes complete lysis of a fixed dose of cells—in this case 0.1 cc. of a 10 per cent suspension—in the presence of a sufficient excess of alexin. This unit varies, of course, as does the upper limit of the hemolytic zone, with the degree of resistance of the cells. The first step, then, is the determination of the hemolysin unit—a procedure that ordinarily need not be



repeated oftener than once in two weeks. This unit, being known, small amounts of four, or more, dilutions of hemolysin are prepared so as to contain in series from 1.3 to 4, or more, units per 0.1 cc., thus surely including the upper zone limit. To these dilutions are added correspondingly equal amounts of 10 per cent cell suspension and thorough admixture is effected by immediate shaking. These dilutions of sensitized cells, in a dose of 0.2 cc., are used for filling four, or more, separate but

TABLE 1  
*Simultaneous alexin and hemolysin titrations*

ALEXIN 1 PER CENT	SALT SOLUTION	SENSI- TIZED CELLS	TEN PER CENT CELL SUSPENSION SENSITIZED WITH AN EQUAL VOLUME OF FOLLOWING DILUTIONS OF HEMOLYSIN*							
			Dilution:							
			100	200	300	400	500	600	700	800
			Units:							
			10.0	5.0	3.3	2.5	2.	1.7	1.4	1.25
cc.	cc.	cc.	Hemolysis:							
			C	C	C	C	C	C	C	S
			C	C	C	C	C	C	S+	S
			C	C	C	C	C	C	S	S
			S+	S+	S+	S+	S+	S+	W+	W
			S	S	S	S	S	S	W	W

C = Complete hemolysis.

S+ = Very strong hemolysis.

S = Strong hemolysis.

W = Weak hemolysis.

\* Hemolysin titer = 0.0001 cc.

identical alexin titrations, which are read after one hour in the water-bath at 37°-38°C. The advantages of using this hemolytic zone limit are that the varying resistances of different lots of sheep cells, or of the same lot at different times, are compensated for by the use of more or less hemolysin; a very accurately balanced system is secured; and, by titrating the two active factors simultaneously, considerable time and manipulation are conserved.

A specimen protocol will make the method more easily understood.

In the above titration the upper limit of the hemolytic zone for the cells used lies between the 1 to 600 and 1 to 700 dilutions, and 600 is chosen as the proper dilution with which to sensitize cells for tests. The hemolysin titer, 0.0001 cc., had been determined previously with an excess of alexin, 0.02 cc. The largest amount of alexin, 0.008 cc., used in the above titration is not a sufficient excess with which to determine the minimum hemolytic dose of hemolysin. The "unit" of alexin, as terminated by this method, in the titration instanced is 0.006 cc.—the amount that produces complete lysis of the cells sensitized with the hemolysin dilution which marks the upper limit of the hemolytic zone. One and one-half units of alexin have been used in all serum and antigen titrations.

*Cross-fixations.* The plan of conducting the cross-fixation tests has been first to titrate the immune sera with one-quarter of the anticomplementary dose of their homologous antigens; to titrate all antigens with approximately two "preliminary" units of homologous and heterologous immune sera; then to titrate all sera with approximately two units of homologous and heterologous antigens. A "preliminary" serum unit is the smallest amount which gives complete inhibition of hemolysis with one-quarter of the anticomplementary dose of homologous antigen. An antigen unit is the smallest amount which gives complete inhibition of hemolysis with approximately two preliminary units of homologous immune serum. A "final" serum unit is the smallest amount which gives complete fixation with approximately two homologous antigen units. Approximately two of these final units have been used in the titration of those antigens for which no immune sera had been prepared. The dosage is stated as "approximately" two units because, owing to the extreme delicacy of the alexin fixation reaction, it is difficult to obtain precisely consistent results. Factors which give rise to minor variations are the temperature of the ice-box, the time elapsed before the tests are read, the resistance of the sheep erythrocytes, the age and potency of the immune sera, conditions of illumination when the tests are read, etc. The determinations of the autoinhibitive dose of antigen and of the pre-

liminary unit of serum have been made immediately before each occasion that cross-titrations were to be carried out, in order to make adjustment for the gradual loss in antibody content of the serum and for differences in fixability of different lots of alexin.

The technic of titrations, in general, is that described in "Pathogenic Micro-organisms" by Park and Williams, 1914, pp. 184-187, with the exception that the total volume of all tests is 1 cc.—one-fifth that of the classical Wassermann. Only complete inhibition of hemolysis—the supernatant fluid being water-clear without the slightest perceptible yellow tint after the tests have stood over night in the ice-box at 10°C., has been taken as the criterion of fixation.

#### AGGLUTININ-ABSORPTION EXPERIMENTS

Experiments upon the specific absorption of agglutinins as a means of detecting antigenic differences have thus far been subordinated to the work with alexin-fixation, chiefly because of the burdensome task of preparing the vast quantities of pure bacterial suspensions, which are necessary for absorbing sera. For this reason, also, the various sera to be absorbed are diluted 1-50 or 1-100, depending upon their agglutinin titer, in order that less of the suspension need be used for complete removal of specific strain agglutinins.

The technic has been as follows: to a suitable amount of serum dilution is added an equal amount of concentrated suspension (5 cc. to 10 cc. per Blake bottle), the mixture is incubated for one hour; then it is centrifuged until the bacteria are sedimented, and the supernatant fluid is removed. This fluid is immediately titrated with the absorbing organism to determine whether or not all of the agglutinins for that strain have been removed, and, as agglutination by absorbed serum is rather slow, this test is left in the water-bath over night. The remainder of the absorbed serum is left in the ice-box to be re-absorbed, or to be tested with other strains as may be indicated by the results of the preliminary test. The macroscopic test has been used, the volume of serum dilution being 1 cc., the dilution being

progressively multiplied by two, and 0.05 cc. of formolized bacterial suspension being added to each tube. Controls of 1 cc. of physiologic saline plus 0.05 cc. of suspension have always been employed, but spontaneous agglutination has never been observed.

The great obstacle to correct interpretation of agglutination tests has been the fact that different strains are inherently different and somewhat variable in their agglutinabilities. This has been overcome by setting up the following control with each test. An equal amount of 0.1 per cent formolized salt solution is added to a portion of the original serum dilution and the mixture is treated in the same way, except for centrifuging, as are the other tubes containing serum that is being absorbed. With suitable dilutions of this unabsorbed serum the agglutination limits of each of the strains to be tested is determined simultaneously with its limits with the absorbed serum. Only in this way can one be sure that a strain that is agglutinated by an absorbed serum is so agglutinated because it possesses specific agglutinogens, which correspond to the specific agglutinins remaining in the absorbed serum, and not because of its own high agglutinability.

It is difficult to obtain significant results when a relatively inagglutinable strain is used as the absorbing agent unless a considerable excess of suspension is used—more than is necessary merely to absorb the agglutinins for that particular strain. This, however, is probably not due to a weaker absorptive power of inagglutinable strains in general (21), but rather to the fact that the preliminary titration of the absorbed serum with an inagglutinable strain does not, under such conditions, afford a reliable index of absorption.

Early in this work it was observed that a certain absorption experiment first performed with fresh serum, when repeated a month later with the same serum, meanwhile stored in the ice-box, gave negative results in the second test. It was suspected that the minor agglutinins, which are probably responsible for positive findings after absorption, had so depreciated with the lapse of time that they no longer appeared in the serum dilutions used.

TABLE 2  
*Serum titrations*

	SERUM STRAINS	ANTIGEN STRAINS											
		48	74	75	94	95	5	7	13	1	3	4	9
Group I.....	48	+			+	+		+	+			+	
	74		+	+		+			+	+	+		+
	75		+	+		+	+		+	+	+	+	+
	94	+			+	+	+	+	+	+		+	+
	95	+	+	+	+	+		+	+			+	+
Group II.....	5		0	0	0		+	+	+	+	0	+	0
	7	0			0	0	+	+	+				
	13	0	0	0	0	0	+	+	+	+	0	+	0
Group III.....	1		+	+	0		+		0	+	+	+	+
	3		+	+			+		0	+	+	+	+
	4	0		+	0	0	+		+	+	+	+	+
	9		+	+	0	+	+		+	+	+	+	+

+ Denotes complete fixation in approximately as small a dose with heterologous as with homologous antigen.

0 Denotes complete or partial fixation with heterologous antigen only with three or more times the minimum dose which, with homologous antigen, gives complete fixation.

The blanks indicate that no tests were made.

TABLE 3  
*Antigen titrations*

	ANTI-GEN STRAINS	SERUM STRAINS											
		48	74	75	94	95	5	7	13	1	3	4	9
Group I.....	48	+			+	+		0	0			0	
	74		+	+		+	+		0	+	+		+
	75		+	+		+	0		0	+	+	0	+
	94	+			+		0		0	0		0	0
	95	+	+	+	+	+		0	0			+	+
Group II.....	5		+	+	+		+	+	+	+	+	+	+
	7	+			+	+		+	+				
	13	+	0	0	+	+	+	+	+	+	+	+	+
Group III.....	1		+	+	+		+			+	+	+	+
	3		+	+						+	+	0	+
	4	+		+	+	+	+		+	+	+	+	+
	9		+	+	+	+			0	+	+	+	+

+ Denotes complete fixation in approximately as small a dose with heterologous as with homologous serum.

0 Denotes complete or partial fixation with heterologous serum only with three or more times the minimum dose which, with homologous serum, gives complete fixation.

TABLE 4  
*Agglutinin-absorption*  
 Serum 802 > 13 (Group II)

SERUM DILUTION	AGGLUTINATES STRAINS											ABSORBED WITH
	1	3	5	7	13	23	27	49	74	75	94	
125	0	±	0	0	+	0	0	0	±	±	+	Strain 4 (Group III)
250	0	0	0	0	0	0	0	0	0	0	+	
500	0	0	0	0	0	0	0	0	0	0	0	
125	} Negative throughout											Strain 5 (Group II)
250												
500												
125	0	0	+	+	+	0	0	0	0	0	0	Strain 94 (Group I)
250	0	0	+	+	+	0	0	0	0	0	0	
500	0	0	0	0	+	0	0	0	0	0	0	
1,000	+	+	+	+	+	+	+	+	+	+	+	Control of serum treated with equal part of formalin saline solution
2,000	+	+	+	+	+	+	+	+	+	+	+	
4,000	0	+	+	+	+	+	+	+	+	+	+	
8,000	0	+	+	+	+	±	±	±	±	+	+	
16,000	0	±	±	±	±	±	±	±	±	±	+	
32,000	0	0	0	0	0	0	0	0	0	0	0	

TABLE 5  
*Antigen titrations with two final serum units*

ANTIGEN	SERUM STRAINS		ANTIGEN	SERUM STRAINS		ANTIGEN	SERUM STRAINS	
	13	94		13	94		13	94
1	0	0.0012	38	0	0.0016	102	0	0.0016
2	0.004	0.004	46	0	0.0016	109	0	0.004
3	0.006	0.0016	47	0	0.0012	110	0	0.004
4	0.006	0.004	48	0	0.0016	112	0	0.0016
5	0.006	0.006	49	0	0.0008	113	0	0.004
6	0	0.0016	50	0	0.002	117	0	0.0008
7	0.006	0.004	57	0	0.006	CP	0	0.002
9	0	0.0012	59	0	0.006	CT	0	0.0016
10	0	0.008	60	0	0.002	Enteritidis	0	0
12	0.006	0.004	61	0	0.008	Alkaligenes	0	0
13	0.004	0.004	66	0	0.004	Supestifer	0	0
14	0.004	0.002	67	0	0.0008	T. murium	0	0
15	0	0.004	70	0	0.0008	Fowl typhoid	0	0
16	0.008	0.004	72	0	0.004	Paratyphosus A	0	0
17	0	0.004	73	0	0.002	Paratyphosus B	0	0
18	0	0.0016	74	0	0.004	Coli A	0	0
19	0	0.008	75	0	0.004	Coli B	0	0
20	0	0.008	78	0	0.004	Dys. Flexner	0	0
23	0	0.0016	79	0	0.006	Dys. Hiss Y	0	0
27	0	0.004	90	0	0.008	Dys. Shiga	0	0
32	0	0.004	94	0	0.002	Morgan I	0	0
35	0	0.002	95	0	0.002			

Decimal figures denote the smallest amounts of antigen which give complete fixation with two final units of serum strains, 13 (Group II) and 94 (Group I).

Accordingly, fresh, unheated sera were used after this for all absorption experiments.

It was also suspected that the Danysz phenomenon (6) might, as in the interaction of many colloids, here be operative. Consequently the bacterial suspension, in some instances, has been added in fractional doses in order that the absorption might be effected with more economy of material. No definite comparative data on this point are available at this time.

#### DISCUSSION

Tables 2 and 3 are composites of the results of antigen and serum cross-titrations which have in nearly every instance been confirmed by several repetitions, sometimes with sera from different rabbits. It will be noted that there appears to be a greater disparity between strains as indicated by antigen cross-titrations than as indicated by serum cross-titrations. This is probably due to the variability of preliminary serum units, a variability which is, in turn, due to the different fixation indices of the antigens with which these units were obtained. For example, a preliminary serum unit found by titrating against ten antigen units (fixation index = 40) will be larger and correspondingly will fix with more antigens, and in a less specific manner, than will a serum of which the preliminary unit has been found by titrating against fifty antigen units (fixation index = 200). For this reason the results of antigen cross-titrations are not very closely comparable. Had the development of this investigation been such as to allow it, the method of standardizing all sera against one highly polyvalent antigen would also have been followed, although it would have been impossible correctly to balance such an antigen because of our lack of knowledge concerning the serologic reactions of all the strains.

In the serum cross-titrations, however, a somewhat more proportional relation between antibody content and antigenic value has been obtained; hence the results are far more regular. On the basis of these results the strains of typhoid bacilli so far examined are tentatively separable into three groups. Group I

is made up of strains whose antisera cross-fix with all other strains; Group II strains cross-fix with each other and with some members of Group III but not with Group I; Group III strains are more heterogeneous and are irregular in their cross-fixations. The larger number of strains so far studied falls in Group I, and these strains are of rather recent isolation.

The results of a more extended study of this phenomenon appear in table 5. Antigens prepared from all strains were titrated with two final serum units of two sera immune respectively to strain 13 (Group II) and to strain 94 (Group I). Antigens prepared from allied organisms were included in this experiment in order to establish the specificity of the results obtained under our technical conditions.

The table shows that serum strain 94 fixes with all our typhoid antigens; that serum strain 13 fixes with none of the strains which have been under artificial cultivation for less than one year; and that a number of the older laboratory strains—1, 6, 9, 10, 15, still retain a characteristic of Group I strains inasmuch as they do not give fixation with a serum immune to a Group II strain.

It does not seem unreasonable then to assume that fresh strains ordinarily possess a greater number of antigenic complexes—since they cross-fix not only with themselves but with old strains as well—than do those that have long been under artificial cultivation. Group II is chiefly made up of very old strains,<sup>4</sup> including “Hopkins” and “Rawlings,” which seem to be of less antigenic complexity. These circumstances would seem to furnish a cogent argument for the use of only recent strains for purposes of protective immunization. When, however, some of the agglutinin-absorption protocols are examined it becomes evident that the problem is far from being so simple.

<sup>4</sup> Our historical notes on strain 7, which is in Group II, indicate that it is the same as strain 3, which is in Group III. The sub-culture of 7 was obtained a year later, however, and there is a possibility that it may have been mis-labeled. Possibly, also, different environmental conditions of which we are uninformed may have caused changes in its antigenic properties. Lastly, it is not inconceivable that the original case may have been infected with two different strains, which were accidentally separated by sub-culture.



Table 4 shows that serum 802, immune to strain 13 (Group II) after absorption with strain 94 (Group I), still contains agglutinins for 5, 7, and 13 which are all in Group II. This is not in strict accord with the apparently valid assumption that Group I strains contain all of the typhoid antigenic complexes, unless, of course, one chooses conveniently to postulate their possession of partial antigens in amount sufficient only to give the fixation reaction, but not completely to absorb the agglutinins, both major and minor, from heterologous sera.

Table 4 shows that strain 5 (Group II) absorbed all of the agglutinins from serum 802, as would be expected. Absorption with homologous strains has been found always to remove all of the agglutinins. Strain 4 acts irregularly and the results are considered inconclusive because the control shows that only the highly agglutinable strains give a positive reaction.

Absorption of Groups I and II sera respectively with Groups I and II strains gives rather consistently negative results—that is, all agglutinins are removed. Absorption of Group I sera with a Group II strain usually removes all agglutinins for other Group II organisms, while agglutinins for most of Group I strains are still demonstrable. The reverse is likewise true. Occasionally, however, results have been obtained which indicate the necessity of further examination of the specific absorptive capacities of strains as a possible criterion of differentiation. Some Group I strains have been encountered which do not remove all of the agglutinins from certain Group I sera, and some which do not remove all of the Group I agglutinins from certain Group II sera. With the development of a finer technic and with intensive study on this point, more consistent and convincing results may be obtained. At present, it is considered that these findings are within the range of experimental error and deserve only parenthetical mention. Studies upon Group III strains have advanced only far enough to show that an irregular behavior similar to that shown in alexin-fixation is manifest also in agglutinin-absorptions.

An attempt has been made still further to strengthen the evidence by doing alexin-fixations on absorbed sera, but during ab-

sorption by our technic, enough of the antigen dissolves in the diluted serum so that the absorbed serum alone gives fixation and thus far no differentiation has been possible by this method. Probably, brief absorption at low temperature with *well washed* suspensions would, for this purpose, be superior to our technic.

There remains a residuum of fragmentary and incidental data which will be referred to briefly.

There is no relation between the grouping of the strains recently isolated by Gay and Chickering (9), and their virulence, as indicated by the severity of the disease which they caused, or the toxicity of any of the strains, as indicated by the severity of the reactions that were produced in the course of immunizing rabbits.

Strain 94 (Group I) was almost totally insusceptible to the bactericidal influence of Group I or Group II sera; strain 13 (Group II) plates were sterilized by homologous serum, and showed a marked reduction in the number of colonies when titrated with a Group I serum.

The fact that Group II is made up of old strains gives dialectic warrant to a consideration of the possibility of biologic variations among typhoid strains. Many of our other laboratory strains fall in Group III and some in Group I; hence it would seem that any tendency toward antigenic simplification with age would not be necessarily a general rule but rather a property of individual strains, some losing in antigenic complexity under artificial cultivation, others, not. A comparison of strains grown continuously on blood agar, with the same strains grown on plain agar would be interesting in this connection.<sup>5</sup> Horrocks (13), by repeated animal passage of a strain of *B. typhosus*, was able to induce the characteristics of a streptobacillus, but this strain still produced typical agglutinins, tropins and fixation bodies for *B. typhosus* and absorbed the agglutinins from a known antityphoid serum. Kutscher (15) refers to several investigators who have observed more or less marked morphologic and cultural changes in strains of *B. typhosus*, but no instance of serologic variation is mentioned.

<sup>5</sup> An antigen prepared from a subplant of No. 13 grown for fifteen "generations" on blood-agar did not, however, fix with a Group I serum.

A comparison of the sera, taken after only four or five injections, with the sera taken months later from the same animals after they had received ten to fifteen injections, affords some indication that the degree of immunization determines the range of reaction. Otherwise stated, the serum from a prolongedly immunized animal did not show so great a difference between the limits of fixation with homologous and heterologous antigens. This is confirmatory of the observations of Magnus (22) who worked with the precipitin reaction in a taxonomic study of various members of the *Gramineae* family. The same phenomenon has been observed in studies on pure vegetable proteins (18). It suggests at once the advisability of doing all cross-titrations with sera which have been produced with but few inoculations. No difference was noted regarding the relative stability of specimens of such sera, as was reported by Coca (5), who found that hemolytic properties were much more stable in the sera of rabbits that had received but few injections of erythrocytes.

#### SUMMARY AND CONCLUSIONS

By means of the alexin-fixation reaction it has been found possible to demonstrate consistent antigenic differences among some strains of *B. typhosus*. In accordance with these differences a number of strains have been tentatively allocated in three fairly well-defined groups. Group I is made up of strains of rather recent isolation; no strain in this group has been under artificial cultivation for more than two years. Older strains, isolated from three to fifteen years ago, compose Group II. The third group is made up of those strains that fall neither in Groups I nor II, and that cross-fix irregularly with each other and with the members of the other groups. Group III strains are all more than three years old.

By means of agglutinin-absorption experiments, findings have been obtained that, in general, harmonize strikingly with the results of cross-fixation. This confirmatory evidence, secured by a widely different serologic method, tends strongly to prove that these antigenic differences among typhoid strains, as

demonstrated by alexin-fixation, represent an actual condition and are not fortuitous.

It is considered that the evidence of antigenic differences thus far discovered among different strains of *B. typhosus* is sufficiently valid to warrant the presentation of these data, and sufficiently encouraging to justify extension of this work, especially with regard to the comparative protection afforded by sera obtained by immunization with strains of different character.

The facts that a serum immune to any recently isolated strain cross-fixes with all other strains, old or young, while sera immune to older strains do not so cross-fix, would lead to the seemingly justified assumption that only do the young strains contain all of the antigenic complexes of typhoid bacillary protein. The logical conclusion, then, would be that a young strain ought to afford the most efficient protection when used for prophylactic immunization against typhoid fever. However, the results of the work on agglutinin-absorption, if correctly interpreted, do not warrant the assumption that a univalent vaccine is sufficient. Therefore, although the grouping of strains here given is but tentative, it seems advisable, for the present, to recommend the use of a balanced polyvalent typhoid vaccine, for immunizing and therapeutic purposes, compounded in accordance with these groups. The experimental work here set forth distinctly does not uphold the prevailing practice of employing a single old strain for prophylactic immunization against typhoid fever.

It is a pleasure here to acknowledge my gratitude to Dr. F. P. Gay for the helpful interest he has taken in this work.

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# THE HECHT (GRADWOHL MODIFICATION) COMPLEMENT FIXATION REACTION IN SYPHILIS WITH SPECIAL REFERENCE TO CHOLESTERINIZED ANTIGENS<sup>1</sup>

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Various modifications of the Wassermann reaction utilizing the complement and natural antishoop hemolysin of human serum, have been advocated from time to time for the purpose of conducting the test in a more economical manner or, more particularly, to increase its delicacy and obviate certain sources of error ascribed to the inactivation or heating of the serum.

Of these methods, that of Hecht (1, 2 and 3) received considerable attention. As originally proposed and with later modification, this test employs an alcoholic extract of guinea-pig heart, human fetal heart and other extracts as antigen using 1 cc. of 1:50, 1:100 and 1:200 dilutions with a constant dose of 0.1 to 0.2 cc. of fresh active serum. The fourth tube of the series serves as the control. After a primary incubation of a half to one hour, 1 cc. of a 2 per cent suspension of sheep cells is added to all tubes followed by re-incubation for one to two hours.

No directions are given for determining the proper dose of antigen and the dose of corpuscles is purely arbitrary and based upon the observation that the great majority of human sera in dose of 0.1 cc. contain sufficient natural antishoop hemolysin and complement to hemolyse this number of cells. In the event this does not prove true with an individual serum, as indicated by a lack of hemolysis in the serum control tube, the test is worthless.

<sup>1</sup> Presented before the meeting of the American Association of Immunologists, May, 1916.

Gradwohl (4) has modified this technic in a manner calculated to remove this source of error. A preliminary titration of the natural antishoop hemolysin is made of each serum to determine the dose of corpuscles to be used. Into each of a series of 10 test tubes he places 0.1 cc. of serum, increasing amounts of a 5 per cent suspension of sheep cells ranging from 0.1 cc. to 1 cc. and sufficient normal salt solution to make the volume equal in all. After incubation in a water bath for half an hour the "hemolytic index" is read off for each serum, that is, the largest amount of corpuscle suspension that is just completely hemolysed. In the main test which is conducted with 0.1 cc. serum and three strengths of antigen, about one-third the amount of corpuscle suspension indicated in the index, is employed. Details regarding the preparation of antigen, its titration and the doses employed were not given.

In the event of the preliminary titration showing that a serum contains insufficient complement or hemolysin or both, guinea-pig serum complement or an immune hemolysin is added and the test conducted in the usual manner. Particulars regarding the amounts of these to be added were not stated.

Gradwohl has found that the sera of 98 per cent of persons contain some antishoop hemolysin. In his test if a serum has an index below 3, that is, 0.1 cc. serum fails to hemolyse at least 0.3 cc. of a 5 per cent suspension of cells, the results are regarded as of doubtful value; if the index is above 3, that is, permits the use of a larger dose of cells, the results are regarded as absolute.

Hecht claims on the basis of a large number of comparative tests with the Wassermann reaction, that his method yields 10 per cent more positive reactions than the latter; Gradwohl has also found in a large number of comparative tests, that the Hecht reaction with his modification is more delicate and a valuable control on the Wassermann reaction.

My own experience with the original Hecht reaction lead me to the conclusion that it was subject to the error of yielding falsely positive reactions and particularly when crude alcoholic extracts of syphilitic liver or heart were used as antigens. In



this manner I have found it subject to the same error of false complement fixation as the Noguchi test employing active serum and to which Noguchi has carefully drawn attention and designated as a "proteotropic reaction." In this connection I have found suitable extracts of the acetone insoluble lipoids of heart muscle best suited for the Hecht test, because least likely to yield these false complement fixation reactions.

Like the Noguchi test with active serum, I found the Hecht reaction on the other hand to be quite delicate and of service as a control on the Wassermann reaction more particularly in a negative way, that is, a negative reaction with active serum under proper conditions, being better evidence of the absence of syphilis or its cure, than a negative Wassermann reaction conducted with an alcoholic extract of syphilitic liver. In my experience, however, the employment of *suitable* alcoholic extract of heart muscle re-enforced with cholesterin, has greatly increased the delicacy of the Wassermann reaction (3, 4) and in a manner approaching the delicacy of the Hecht and Noguchi (with active serum) reactions.

In order to test out these conclusions and more particularly to determine the value of the Hecht reaction as compared with the Wassermann reaction conducted with an antigen of an alcoholic extract of heart muscle re-enforced with cholesterin, I have conducted comparative tests with both systems and with three different antigens in each; the former reaction being conducted with the Gradwohl modification of determining the hemolytic index of each serum.

#### TECHNIC AND METHOD OF STUDY

*Sera.* The sera of 360 persons were subjected to both tests at the same time. An effort was made to collect these sera within forty-eight hours of the time of the tests in order to ensure the presence of active complement. A number of sera, however, were purposely collected at longer intervals and a number from various cities distant from Philadelphia were included in the series in order to study the reactions under varying circumstances.

*Wassermann reactions.* These were conducted with the same three antigen-extracts as used in the Hecht-Gradwohl reactions. The anticomplementary and antigenic units of each as determined at frequent intervals with 0.2 cc. inactivated normal and syphilitic sera respectively, were as follows:

Antigen "C. H." (cholesterinized alcoholic extract of human heart); anticomplementary unit (1:20) 2.5 cc.; antigenic unit (1:20) 0.05 cc.; antigenic dose (1:20) 0.2 cc. The dose employed was therefore four times the antigenic unit and this amount was at least twelve times less than the anticomplementary dose.

Antigen "S" (alcoholic extract of syphilitic liver); anticomplementary unit (1:10) 1.8 cc.; antigenic unit (1:10) 0.1 cc.; antigenic dose (1:10) 0.3 cc. The dose employed was three times the antigenic unit and this amount was at least six times less than the anticomplementary unit.

Antigen "A" (acetone insoluble lipoids of human heart); anticomplementary unit (1:20) 2.2 cc.; antigenic unit (1:20) 0.03 cc.; antigenic dose was 0.1 cc. of 1:20 dilution which was slightly more than three times the unit and this amount at least twenty times less than the anticomplementary unit.

All reactions were conducted with inactivated sera in dose of 0.2 cc. with each antigen; 1 cc. of a 1:20 dilution of mixed guinea-pig serum complement (=0.05 cc. undiluted serum); two units of antishoop hemolysin as titrated each time with the complement and 1 cc. of a 2.5 per cent suspension of washed sheep cells. Tests and controls were incubated for one hour at 37°C., re-incubated for the same period after the addition of hemolysin and cells and the results read immediately when the controls showed complete hemolysis.

*Hecht-Gradwohl reactions.* The anticomplementary and antigenic units of the three antigens were determined at frequent intervals in a series of titrations employing the mixed fresh sera of normal and syphilitic persons respectively, in dose of 0.1 cc. The hemolytic indices of these mixed sera were determined beforehand.

The *anticomplementary titrations* were conducted by placing

in a series of test tubes a constant dose of 0.1 cc. of the fresh and mixed sera of two healthy, non-syphilitic and Wassermann negative persons, and increasing doses of the antigens diluted with normal salt solution; normal salt solution was added to make the volume of each tube about 1.5 cc. After incubation for an hour at 37°C. the proper dose of cells was added according to the hemolytic index and the tubes were re-incubated for an hour after which the results were read. The smallest dose of each antigen giving slight inhibition of hemolysis was taken as the anticomplementary unit.

The antigenic titrations were conducted in the same manner with 0.1 cc. of the mixed and fresh sera of two known syphilitics. The unit was taken as the smallest amount of antigen yielding complete inhibition of hemolysis; the antigenic dose was double the unit and these amounts were from five to six times less than the anticomplementary units.

The results of a single set of these titrations are shown in table 1.

These titrations are of great importance because the activity of human serum is quite sensitive to the inactivating influence of tissue extracts. For this reason the margin between the anticomplementary and antigenic units is less than when the same antigens are titrated with guinea-pig serum complement. Certainly no antigen should be employed in this test without preliminary titration *with human sera* to determine its antilytic properties and antigenic sensitiveness and the above method has given me satisfactory results.

In my experience I have frequently found by titrations that the proper doses of antigens for the modified Hecht-Gradwohl test were the same as those found proper for the Wassermann reaction with five times more dilution. For example, the dose of my cholesterinized heart antigen in the Wassermann reaction was 0.2 cc. of a 1:20 dilution and for the Hecht-Gradwohl test, 0.2 cc. of a 1:100 dilution.

The hemolytic index of each serum was determined after Gradwohl's method except that I used but five tubes instead of ten for this purpose, as I have not found any sera in dose of

0.1 cc. capable of hemolysing more than 0.5 cc. of a 5 per cent suspension of washed sheep cells prepared after the last washing with prolonged centrifugation. Also instead of using three doses of the same antigen I used one dose of the three different antigens named above.

TABLE 1  
*Antigen titrations for the Hecht reactions*

(A) ANTICOMPLEMENTARY					(B) ANTIGENIC				
Antigen	Known normal sera*	Antigen			Antigen	Known syphilitic sera†	Antigens ‡		
		C. H. 1:100	S 1:50	A 1:100			C. H. 1:100	S. 1:50	A. 1:100
	cc.				cc.	cc.			
0.2	0.1	H§	H	H	0.05	0.1	++	+	+
0.3	0.1	H	H	H	0.1	0.1	++++	++++	++
0.4	0.1	H	H	H	0.15	0.1	++++	++++	++++
0.6	0.1	H	H	H	0.2	0.1	++++	++++	++++
0.8	0.1	H	H	H	0.25	0.1	++++	++++	++++
1.0	0.1	H	H	H	0.3	0.1	++++	++++	++++
1.2	0.1	S.I.H.	H	H	0.35	0.1	++++	++++	++++
1.5	0.1	M.I.H.	S.I.H.	S.I.H.	0.4	0.1	++++	++++	++++
Serum control	0.1	H	H	H	Serum control	0.1	—	—	—

\* Mixed sera of two persons: index 0.2 cc. cells.

† Mixed sera of two syphilitics: index 0.3 cc. cells.

‡ Antigenic dose of "C.H." (cholesterinized alcoholic extract of heart: 0.2 cc. of 1:100 dilution.

Antigenic dose of "S" (alcoholic extract of syphilitic liver): 0.2 cc. of 1:50 dilution.

Antigenic dose of "A" (acetone insoluble lipoids): 0.2 cc. of 1:100 dilution.

§ H = complete hemolysis.

S.I.H. = slight inhibition of hemolysis.

M.I.H. = marked inhibition of hemolysis.

Briefly stated my tests were conducted as follows:

Nine small sterile test tubes (10 by 1 cm.) were arranged for each serum and properly labeled; into each was placed 0.1 cc. of fresh unheated serum. To the first five tubes were added respectively 0.1, 0.2, 0.3, 0.4 and 0.5 cc. of a 5 per cent suspension of sheep cells; to the sixth, seventh and eighth tubes were added the proper antigenic doses of antigens C. H., S and A

respectively. The last or ninth tube served as the serum control. Sufficient normal salt solution was added to each tube to bring the total volume to 1.0 cc.

After one hour's incubation at 37°C. the hemolytic index was read on each serum and *one-half* the indicated doses of cells added to the remaining four tubes of each series. After incubation for one-half to one hour according to the hemolysis of the controls, the results were read and recorded after the same —, +, ++, +++, and +++++ method as in the Wassermann reaction.

Hemolysis is usually quite prompt and active, and it is well to read the results as soon as the controls show complete hemolysis.

As stated above, the ninth tube of each series is the serum control. The antigen controls were prepared with the fresh active serum of healthy laboratory assistants which also served as negative controls; owing, however, to the difficulty of obtaining fresh serum from a syphilitic person twice each week, positive controls were not always included. Since ten or twenty or more sera from varied sources were tested each time a sufficient number of syphilitic sera were always included to satisfy me regarding the antigenic sensitiveness of the extracts.

In case a serum failed to hemolyse completely at least 0.1 cc. of cells, guinea-pig serum complement or hemolysin or both were added. If the serum was over forty-eight hours old I assumed that lack of hemolytic activity was due to the absence of complement and activated the last four tubes of the series by the addition of 0.5 cc. of a 1:20 dilution of guinea-pig serum complement followed by reincubation for an hour and the addition of 0.1 cc. of cells. If hemolysis did not occur or was incomplete, I concluded that the serum was either anticomplementary or lacked natural antisheep hemolysin and did nothing further.

*All of the tests recorded in this study, however, were those in which the sera contained complement and hemolysin and yielded a hemolytic index, because the addition of an alien complement or immune hemolysin removes the reaction from the category which is under consideration.*

## RESULTS

*Hemolytic indices.* The hemolytic indices of 360 sera collected in from a few to seventy-two hours before titration are shown in table 2.

TABLE 2  
*Hemolytic indices of 360 human sera*

AGE OF SERUM	DOSE OF SERUM	PERCENTAGE GIVING COMPLETE HEMOLYSIS				PERCENT-AGE GIVING NO HEMOLYSIS	PERCENT HEMOLYSING 0.1 CC. OR MORE CELLS
		With 0.1 cc. cells	With 0.2 cc. cells	With 0.3 cc. cells	With 0.4 cc. cells		
24-72 hrs.	0.1 cc.	31	33.5	19	9	8	92

As shown in the above table, 92 per cent of sera contained sufficient complement and hemolysin to hemolyse at least 0.1 cc. of cells. When the index was 0.1 cc. I have conducted the tests with 0.05 cc. of cells, which is sufficient to indicate a positive or negative result, but not sufficient to indicate varying degrees of complement fixation. As previously stated, Gradwohl has found that the sera of 98 per cent of persons are actively hemolytic and I believe his indices are generally higher than mine. These results, of course, depend upon the concentration of the corpuscle emulsions and will vary with different workers, but are of no practical importance provided the index of each serum is determined and the same corpuscle suspension is used in the complement fixation portion of the test.

*Comparative results with the Wassermann and Hecht-Gradwohl reactions with 360 sera.* These results may be summarized according to my interpretation of the reactions in both tests with the *three* antigens, as follows:

1. The same results in both tests were observed with 295 or 82 per cent of sera. With 120 or 40 per cent of these 295 sera the results were positive in both; with 175 or 60 per cent of sera the results were negative in both tests.

2. The results varied with 65 or 18 per cent of sera and as follows:

(a) A *negative* Wassermann and a *positive* Hecht-Gradwohl reaction occurred with 59 sera or slightly over 16 per cent of the series of 360 sera.

(b) A *positive* Wassermann and a *negative* Hecht-Gradwohl reaction occurred with 6 sera or slightly less than 2 per cent of the series of 360 sera.

I fully expected to find a higher percentage of positive reactions with the modified Hecht technic, because of my former results and the greater delicacy of the hemolytic system and greater sensitivity of human complement to the inactivating influence of tissue extracts.

For these same reasons I am at a loss to explain the instances in which the Wassermann reaction was positive and the Hecht reaction negative. I can only state that I feel quite sure the sera were not anticomplementary as the hemolytic indices ran from 0.2 to 0.4 cc. of cells. All of the sera were from persons known to be syphilitic and undergoing active treatment largely with arsenobenzol. The reactions occurred at different times when ten or more sera were included in a series and with the same antigens used in the same amounts; for these reasons I am not inclined to ascribe the results to an anticomplementary action of the sera or antigens. Theoretically, however, these results can only be explained at present on the basis of manipulative error, unless it is shown later that they were due to biological phenomena at present unknown.

*Analysis of the cases yielding a negative Wassermann and a positive Hecht-Gradwohl reaction.* As stated above this result occurred with 59 sera or slightly more than 16 per cent of the 360 sera. These figures have reference only to our interpretation of the results with all antigens including the cholesterinized extract and in regard to the absolute result without reference to the degree of complement fixation.

It is impossible accurately to analyse this number of cases according to the histories and clinical diagnoses, because the sera were received from varied sources; just as serologists vary in their reports on the same serum so do clinicians vary in their opinions on the same case and what they regard as syphilitic or non-syphilitic symptoms and lesions.

According to the analysis of a smaller number of cases belonging to this group and principally those from the clinic of

my colleague Dr. Jay F. Schamberg, and the results of tests with the sera of laboratory assistants and medical students, I believe that when the Hecht-Gradwohl reaction is positive and the Wassermann reaction is negative, the former is the correct result in the majority of instances and indicates the greater delicacy of the Hecht reaction even when a cholesterinized extract is used in the Wassermann reaction.

On the other hand, I have reason to believe that the modified Hecht reaction yields about 4 per cent falsely positive reactions. These false reactions have been found to occur more particularly with the alcoholic extract of liver and the cholesterinized extract of heart than with the extract of acetone-insoluble lipoids.

*Analysis of the results with reference to the antigens.* In so far as the Wassermann reaction is concerned, the results observed with the three different antigens were quite similar to those previously reported (5, 6). About 10 per cent of the positive Wassermann reactions occurring in this series of 360 cases, were positive only with cholesterinized extract and an analysis of these cases which were mostly from the clinic of Dr. Jay F. Schamberg, showed that they were with few exceptions clinically well defined cases of syphilis undergoing active treatment with arsenobenzol. In my experience it is practically a rule for the reactions with the serum of a syphilitic undergoing active treatment, to become negative first with the alcoholic extract of syphilitic liver, next with the extract of acetone-insoluble lipoids and lastly with the cholesterinized extract. In our opinion, treatment is incomplete unless the serum reacts negatively with the latter extract over a period of at least two years; if this happy result cannot be obtained we advise that the patient remain under observation with occasional periods of treatment.

As I pointed out some years ago (5), cholesterinized extracts may yield slight degrees of complement fixation with a small percentage of the sera of apparently non-syphilitic persons. Similar results were observed in a series of cases which I studied with Dr. Schamberg (6). Further experience has taught me, however, that when the reactions are definitely positive with the cholesterinized extracts alone, that in from 80 to 90 per cent



of such instances the serum is from a person in the tertiary or latent stages of syphilis, or more particularly from a syphilitic under the influence of active treatment. More extensive experience with cholesterinized extracts has taught me to place more and more confidence in the results they yield and for several years it has been my custom to conduct the Wassermann reaction with the three extracts mentioned above.

As previously stated, however, the Hecht-Gradwohl reaction in the present series yielded about 16 per cent more positive reactions than the Wassermann reaction, and the results of both were based upon the reactions of all three antigens including the cholesterinized extract. A close analysis has lead me to the conclusion, however, that a number of these positive Hecht-Gradwohl reactions were to the best of my knowledge non-specific, so that in this series it may be stated that the modified *Hecht reaction yielded about 12 per cent more true positive reactions than the Wassermann reaction with cholesterinized extract. When compared with the Wassermann reaction conducted with an alcoholic extract of syphilitic liver the Hecht-Gradwohl reaction yielded about 26 per cent more true positive reactions and about 18 per cent more reactions than resulted with an extract of acetone-insoluble lipoids in the Wassermann system.*

*Analysis of the falsely positive Hecht-Gradwohl reactions.* My conclusions in this phase of the subject are based largely upon tests conducted with the sera of healthy assistants and students in whom the Wassermann reactions were negative with all antigens and the assumption quite reasonable that they were non-syphilitic persons.

Of the 59 persons reacting positively in the modified Hecht reaction and negatively in the Wassermann, 14 or 4 per cent of 360 sera, were regarded as falsely positive Hecht reactions. Of these eight were positive with the alcoholic extract of syphilitic liver alone; four were positive with the cholesterinized heart and syphilitic liver extracts and 2 with all three of the extracts.

My results also indicate that in the Hecht reaction the superior antigenic sensitiveness of the cholesterinized extract is much less in evidence than in the Wassermann reaction. The results

with this extract and the extract of acetone-insoluble lipoids were closely parallel and since the latter are much less likely to yield false reactions I believe that a suitable and titrated extract of acetone-insoluble lipoids constitutes the best antigen for the Hecht reaction either in its original or modified form.

*Practical value of the Hecht-Gradwohl reaction in diagnosis and as a guide in treatment.* The main drawback to the Hecht reaction is that the serum must be perfectly fresh. For this reason the reaction is not well suited for specimens sent from a distance under ordinary conditions and especially if an interval of forty-eight hours or longer elapses between the time of collection of blood and the test. Unless carefully refrigerated the complement quickly deteriorates and thermolabile anticomplementary bodies develop. These changes are especially likely to occur in warm weather and for these reasons I have conducted the Hecht tests each day and the Wassermann tests twice in the week. Specimens which were carefully refrigerated have proven perfectly suitable seventy-two hours after collection, but in my opinion it is better practice to conduct the Hecht-Gradwohl test within forty-eight hours after the collection of blood.

The Hecht-Gradwohl test is certainly a very delicate reaction and is of particular service as a serological guide to treatment and in the serological diagnosis of those occasional cases yielding negative Wassermann reactions with cholesterinized extracts, but whose histories or clinical manifestations or both indicate syphilis.

As previously stated it takes more extensive treatment in syphilis to extinguish the Wassermann reaction conducted with a suitable cholesterinized extract of heart muscle than with an alcoholic extract of syphilitic liver and in many instances still more persistent treatment to extinguish a positive Hecht reaction. In my opinion this is an advantage, for the treatment of syphilis must be most thorough to be efficacious and to prevent late exacerbations and manifestations. Experience has taught me quite conclusively that it is a serious error to regard a person as cured of syphilis on the basis of a negative Wasser-

mann reaction conducted with an alcoholic extract of syphilitic liver alone; negative reactions with a cholesterinized extract are better evidence and persistently negative reactions with the Hecht test properly conducted with standardized antigens, the best serological evidence of cure at our command at the present time.

I have not compared the Noguchi test employing active serum with the Hecht reaction, in a sufficiently large series of cases to present figures of any value on their comparative merits; the results of a small series of comparative tests with an antigen of acetone-insoluble lipoids in both, have shown, however, that both yield similar results, but as compared with the Wassermann reaction employing cholesterinized extracts as antigens, the latter proved somewhat more delicate, besides being even simpler in technic.

#### CONCLUSIONS

1. The Hecht reaction as modified by Gradwohl, and conducted with a properly standardized antigen constitutes a very delicate control over the Wassermann reaction.

2. About 92 per cent of sera consisting mostly of those collected within a period of forty-eight hours at the time of the tests, were found to contain sufficient complement and natural anti-sheep hemolysin for the conduct of the modified Hecht reaction. In those deficient in one or both of these substances guinea-pig complement or immune hemolysin must be used, but the addition of these alters the nature, sensitiveness and value of the reaction.

3. *The modified Hecht reaction possesses a greater negative than a positive value*, because it is open to the error of falsely positive or proteotropic reactions, as obtained in the Noguchi test employing active or unheated serum.

4. The Hecht reaction yielded about 4 per cent of falsely positive or pseudo reactions with one or more of the three different antigens used in this study; the fewest of these pseudo reactions occurred with an extract of acetone-insoluble lipoids.

5. The antigen or antigens employed in the modified Hecht reaction must be carefully standardized at frequent intervals in order to reduce the percentage of pseudo reactions to a minimum.

6. A suitable extract of acetone-insoluble lipoids of heart muscle generally proved the superior antigen for the Hecht-Gradwohl reaction. An alcoholic extract of syphilitic liver proved of least antigenic sensitiveness and yielded the highest percentage of false or pseudo-reactions.

7. The modified Hecht and Wassermann reactions including the use of cholesterinized extracts, yielded similar results in 82 per cent of a series of 360 sera.

8. The modified Hecht reaction yielded about 16 per cent more positive reactions and about 12 per cent more *true* positive reactions than the Wassermann reaction conducted with a cholesterinized extract as antigen; 26 per cent more *true* positive reactions than the Wassermann conducted with an alcoholic extract of liver and 18 per cent more *true* reactions than the Wassermann with an extract of acetone-insoluble lipoids.

9. *The modified Hecht reaction has its greatest value as a serological control in the treatment of syphilis.* During treatment the Wassermann reaction with an alcoholic extract of syphilitic liver is usually extinguished first and after considerable more treatment it is generally extinguished with a cholesterinized extract; the Hecht reaction is usually last to react in a negative manner.

10. Suitable and standardized cholesterinized alcoholic extracts of heart muscle constitute the most sensitive antigens in the Wassermann reaction with which we are familiar and with experience our confidence in their reliability has steadily increased. In our opinion it is a serious error to regard a person as cured of syphilis on the basis of a negative Wassermann reaction conducted with an alcoholic extract of syphilitic liver alone.

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# CORRECT AND INCORRECT METHODS OF PERFORMING THE "DAILY TITRATIONS" FOR THE WASSERMANN REACTION AND OTHER FORMS OF COMPLEMENT FIXATION

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The strength of guinea pigs complement varies greatly. There are in general two different methods employed by different workers to compensate for this varying potency in determining doses of complement and amboceptor for use in the Wassermann reaction as well as in complement fixation with bacterial antigens. These methods are (1) keeping the dose of complement constant and varying the dose of amboceptor from day to day according to the varying strength of the complement, and (2) keeping the dose of amboceptor constant and varying the dose of complement from day to day according to its varying strength. The fact does not seem to have been recognized that these two procedures represent entirely different things and produce different results.

None of the authors gives any reason for adopting the one or the other procedure. Followers of each method are found among those who adhere to the original requirement of a hemolytic system of two units strength, and among those who attempt to reduce the hemolytic strength to one unit above the strength necessary to neutralize the anticomplementary agents present in the tests. Thus the workers may be divided into two groups each with two subdivisions:

I. The workers that use a hemolytic system of *double* strength (or even more) so as to *overwhelm* all the anticomplementary elements.

(1) Those who use a fixed dose of complement (generally 0.1 cc.) with two (or more) units of amboceptor, such as, Wassermann (1), Citron (2), Sachs (3).

(2) Those who use a fixed dose of amboceptor (generally two units as determined by previous titration) with a double laking dose (two units) of complement, such as Müller (6) MacIntosh and Fildes (4), Walker and Swift (5).

II. The workers that *adjust* the hemolytic system to the *anticomplementary* factors so as to have *one* hemolytic unit above what is destroyed by the *anticomplementary* elements.

(1) Those who use a fixed dose of complement (0.1 cc.) and a single laking unit of amboceptor as determined in the presence of the chief *anticomplementary* factor present (namely the antigen), such as Margarete Stern (7).

(2) Those who use a fixed dose (generally two units) of amboceptor and a single laking dose of complement as determined in the presence of antigen alone or of antigen and normal serum, such as Maslakowitz and Lieberman (8), Thomsen (9), Boas (10), Kromayer and Trinchese (11), Thomas and Ivy (12), Graetz (13), Browning and McKenzie (14).

For the reasons detailed elsewhere<sup>1</sup> the present author has believed that those who varied the dose of complement according to its varying strength were following the proper procedure, and that inevitably if the dose of amboceptor only was varied, one was bound, whenever an unusually strong complement was used, to miss a certain number of weakly positive Wassermann reactions. In the present paper are presented a few of a series of experiments in which this is definitely shown to be true.

Separate experiments were performed on each of two plans of determining hemolytic strength, namely the system of using two units and the system of using one unit as determined in the presence of antigen and normal serum. Positive sera were used in twelve graded dilutions from 1-2 up to 1-32 so that certain dilutions would correspond in reacting strength to weakly positive sera. Care was taken to select only positive sera that

<sup>1</sup>Archives of Internal Medicine in press.



contained no natural hemolysin for sheep cells. The tests were done in half or quarter Wassermann quantities, but the protocols are given for the sake of comparison in full Wassermann quantities. Only a few experiments are detailed below but the experiments were repeated a great many times with slight variations in method and regularly with the same result.

The antigen used in most of the experiments was a simple alcoholic extract of beef heart but enough experiments were done with a cholesterinated (0.4 per cent) human heart extract to demonstrate that the type of antigen made no difference.

The complement used was generally the mixed complement of four or five guinea pigs but in certain experiments the sera of several individual guinea pigs were separately titrated and one or two of those which showed a high complement titer were used separately. The amboceptors had been in use for a long while and their average unit, as determined with the fixed dose (0.1 cc.) of a large number of different complements, is what is meant below when the unit of amboceptor is spoken of.

#### FIRST SERIES (TWO HEMOLYTIC UNITS)

Each experiment consisted of setting up two rows of tests with twelve dilutions of a known positive serum. In the first row were used the fixed dose (0.1 cc.) of complement, and two hemolytic doses of amboceptor (as determined by a fresh titration on each occasion). In the second row the dose of amboceptor added was fixed (two units) and the complement represented two units, i.e., twice the least amount that gave complete laking with two units of amboceptor (as determined by a titration immediately before the tests were set up). As controls the double dose of antigen and the undiluted positive serum were separately set up in each instance with the amount of complement and amboceptor used in each row.

It was found that *invariably the second row containing two hemolytic units of complement gave a positive reaction at higher dilutions of the positive serum than the first row containing two hemolytic units of amboceptor.* In the instances where the com-

plement was only of average strength this difference was not very great, but in the instances (such as Experiment 1) in which the complement was stronger than usual the differences were very striking, so that there were several dilutions (which might be taken to represent several different grades of weak positive sera) which gave negative or doubtful reactions with two units of amboceptor (first row) and clear cut positive results with two units of complement (second row).

*First series (two hemolytic units)*

*Experiment I*

PRELIMINARY TITRATIONS	AMBOCEPTOR (1-200)	0.2	0.24	0.28	0.32	0.4	
1	Complement (1-10) 1 c .....		c	c	c	c	Sheep cells were added and readings made after 30 minutes in water bath at 37°C.
	COMPLEMENT (1-10)		0.2	0.25	0.3	0.35	
2	Amboceptor (2 units) 0.6S.....			c?	c	c	C = complete hemolysis.

The unit was taken as 0.27 of complement.

EXPERIMENT		ANTIGEN 1.0 CC. (1-10 DILUTION, ALCOHOLIC BEEF HEART) POSITIVE SERUM DILUTED								
		1-4	1-6	1-8	1-10	1-12	1-14	1-16	1-20	
1	Complement 1.0 cc. Amboceptor 0.5 }	++++	+	0	0	0	0	0	0	(2 units amboceptor)
2	Complement 0.54 Amboceptor 0.6S }	++++	++++	++++	++++	+++	+	0	0	(2 units complement)

After four hours in the ice box at 8°C. Sheep cells were added and the amounts of amboceptor indicated above. Readings after 30 minutes in water bath. +++++, +++, ++, and + indicate complete, almost complete, partial or faint inhibition.

*Experiment II*

PRELIMINARY TITRATIONS	AMBOCEPTOR (1-200)		0.24	0.28	0.32
1	Complement (1-10) 1 cc.....			c	c
	COMPLEMENT (1-10)	0.2	0.25	0.3	0.35
2	Amboceptor (2 units) 0.68.....		c?	c	c

(The unit was taken as 0.27 of complement.)

EXPERIMENT		ANTIGEN 1.0 CC. (1-10 DILUTION) POSITIVE SERUM DILUTED					
		1-4	1-6	1-8	1-10	1-12	
1	Complement (1-10) 1.0 cc..... Amboceptor 0.56... }	++++	0	0	0	0	(2 units amboceptor)
2	Complement 0.54... Amboceptor 0.68... }	++++	++++	+++	0	0	(2 units complement)

## SECOND SERIES (ONE HEMOLYTIC UNIT)

In this series the amount of amboceptor or of complement used was that amount which gave complete laking after having been incubated for one hour in the presence of antigen and of pooled normal human serum containing no natural antishoop amboceptor. This, therefore, does not represent (as the heading suggests), one hemolytic unit, but one hemolytic unit above that amount of complement or amboceptor respectively which is neutralized by the anticomplementary effect of the antigen and of the normal serum.

As in the first series two kinds of titrations were done, the first of amboceptor in the presence of a fixed dose (namely 0.1 cc.) of complement, and the second of complement in the presence of a fixed dose (namely 2 units) of amboceptor. The controls were; (a) the same dose of antigen as used in the tests with the same amount of complement or amboceptor in each case, and (b) the fixed amount (0.2) of the undiluted positive serum.

In this serum control tube the dose of complement or amboceptor of course was smaller than in the test proper and was determined by a separate titration of the complement or amboceptor in the presence of negative serum. (In determining the unit of complement or amboceptor throughout, not the very smallest amount which gave complete hemolysis, but instead the tube containing the next larger amount of complement or amboceptor in the series was taken as the unit to allow for possible slight variations in technic. It seems wise to do this where one uses a bare hemolytic unit instead of an excess as in the first series.

In this series as in the first the results invariably show that *where one hemolytic unit of complement was used fixation was obtained in higher dilutions than where one unit of amboceptor was used.* In the instances where the complement was of average strength as in Experiment II the difference was trifling, and in practice would be negligible. But in the instances where the complement was of somewhat more than of average strength as for instance in Experiment I, the differences are striking and show complete fixation with the single unit of complement at far higher dilutions than with the single unit of amboceptor.

*Second series (using 1 hemolytic unit)*

*Experiment I*

TITRA- TIONS	COMPLEMENT TITRATION (IN PRESENCE OF ANTIGEN AND NORMAL SERUM) AMBOCEPTOR 2 UNITS (0.4) IN ALL TUBES						
	Complement (1-10)		0.5	0.55	0.6	0.65	0.7
1	Antigen 2.0 cc..... } Normal serum 0.2 cc..... }			c	c	c	c
	AMBOCEPTOR TITRATION (IN PRESENCE OF ANTIOEN AND NORMAL SERUM) COMPLEMENT (1-10) 1.0 CC. IN ALL TUBES						
	Amboceptor (1-400)	0.25	0.3	0.35	0.4	0.45	0.5
2	Antigen 2.0 cc..... } Normal serum 0.2 cc..... }		c	c	c	c	c

The sheep cells and amboceptor were added only after complement, serum, and antigen had had a preliminary incubation of 1 hour at 37°C. in water bath.

EXPERIMENT		ANTIGEN 2.0 CC. (1-10 DILUTION) IN ALL TUBES 0.2 POSITIVE SERUM DILUTED							
		1-6	1-8	1-10	1-12	1-14	1-16	1-20	
1	Complement 0.6 cc. Amboceptor 0.4 cc.	++++	++++	++++	++++	+++	+	0	(1 free unit complement)
2	Complement 1.0 cc. Amboceptor 0.32 cc.	++++	++++	+	+	0	0	0	(1 free unit amboceptor)

After 1 hour preliminary incubation at 37°C. in water bath 1 cc. of 5 per cent sheep cells with the amounts of amboceptor indicated were added to each tube. Readings made after 1 hour in water bath at 37°C.

### Experiment II

TITRA- TIONS	AMBOCEPTOR 2 UNITS (0.4)						
	Complement (1-10)		0.6	0.65	0.7	0.75	0.8
1	Antigen 2.0 cc. ....	}	c	c	c	c	c
	Normal serum 0.2 cc.....						
	Complement (1-10) 1.0 cc.						
2	Amboceptor (1-400)		0.4	0.45	0.5	0.55	0.6
	Antigen 2.0 cc.....	}		c	c	c	c
	Normal serum 0.2 cc.....						

EXPERIMENT		ANTIGEN 2.0 (1-10 DILUTION) 0.2 POSITIVE SERUM DILUTED						
		1-8	1-10	1-12	1-16	1-20	1-24	
1	Complement } 0.7 cc. .... } Amboceptor } 0.4 cc. .... }	++++	++++	++++	+++	+	0	(1 free unit complement)
2	Complement } 1.0 cc. .... } Amboceptor } 0.5 cc. .... }	++++	++++	++++	0	0	0	(1 free unit amboceptor)

In these experiments, of course, variations in the fixability of complement play no rôle as no attempt is made to compare one complement with another, but only to compare the two methods of determining hemolytic factors with each complement by itself. For the same reason variations in the strength of the positive serum used on the different dates play no rôle in the result.

#### CONCLUSIONS

The above experiments demonstrate that in the Wassermann reaction the method used by many workers of compensating for increased hemolytic strength of the complement by using a diminished amboceptor dose inevitably leads to a negative result in certain grades of weakly positive sera. *The correct method of compensating for complements of greater than average hemolytic strength is by using a smaller dose of complement.* The method of doing daily complement titrations followed by many workers is greatly to be preferred therefore to the method of keeping the dose of complement fixed and doing daily amboceptor titrations; and undoubtedly the differences between these two methods are partly responsible for the different results that have been reported from different laboratories on identical sera.

The author wishes to acknowledge with thanks the assistance of Dr. Edgar T. H. Tsen.

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# METHODS OF DETERMINING THE OPTIMAL AMOUNT OF "ANTIGEN" FOR THE WASSERMANN REACTION

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In the practical performance of the Wassermann reaction the doses of all the ingredients have to be adjusted to each other. The original Wassermann reaction and many of its modifications follow the principle of employing a fixed dose of complement and as large a dose of antigen as possible without danger of non-specific fixation. Usually those who follow this method also determine the least amount of antigen capable of giving a positive reaction with positive serum (the antigenic unit). They require that the dose actually used shall be a considerable multiple of this unit and state that the more multiples of the smallest antigenic unit contained in the dose used, the better the antigen.

On the other hand there are many authors, such as Browning and McKenzie, Thomas and Ivy, Thomsen, Boas, Stern, who use an arbitrary fixed dose of antigen and adjust the complement to the dose of antigen in such a way that the complement is capable of giving complete hemolysis in the presence of the dose of antigen used. These authors all take rather large doses of the alcoholic extracts, evidently also with the idea that the larger the amount of antigen used the more likely one is to detect weakly positive sera. Since of course, the complement is adjusted in each case to the dose of antigen used, the dose is "safe" in the sense of not giving false positive results.

The present author always subscribed to this opinion, namely, that the larger the dose of antigen that could be used with

safety the better results might be expected, until a chance observation led him to doubt its validity. On investigation he found that when the complement was accurately adjusted to the antigen the rule given above did not hold true at all. The experiment has been repeated a great many times and invariably resulted the same way. Two or three typical examples will be given below.

The titrations and tests were done either according to the method of Thomsen, in which the complement is adjusted to the dose of antigen, or according to the method of Thomas and Ivy in which the dose of complement is adjusted to the antigen and pooled negative serum. A separate titration had to be done therefore to determine the correct dose of complement to be used with each of the doses of antigen experimented with. Each titration consisted of a series of tubes all of which contained the chosen dose of antigen, when the method of Thomsen was followed, and the chosen dose of antigen plus the dose of pooled negative serum, when the method of Thomas and Ivy was followed. The successive tubes in each series contained increasing doses of diluted complement. The volumes of all the tubes were made equal and they were incubated for one hour in the water bath at 37.5°C. Then a dose of sheep's cells sensitized with two units of amboceptor was added to every tube; the tests were returned to the water-bath and readings made at the end of one hour. The least dose of complement that gave complete laking, or a minute fraction above this was used in the tests.

The experiment proper consisted of setting up each dose of antigen with its proper dose of complement in a series of tubes containing progressive dilutions of a known positive serum. These mixtures were incubated in the ice-box for four hours or in the thermostat for one hour, and at the end of this time the sheep cells sensitized with two units of amboceptor were added and the tests were then incubated in the water-bath for an hour. At the end of this time reading of the degree of hemolysis in each tube were made.

The actual experiments were done for the sake of economy in quarter Wassermann quantities, but for uniformity all of them



are reported in the tables as though they had been done in full Wassermann quantities (the doses of all the ingredients merely being multiplied by four). In each instance, as control tests, the undiluted positive serum was set up with one unit of complement (as determined in a separate titration) and each dose of antigen was set up with its appropriate dose of complement. The antigen, an alcoholic beef heart extract, had no hemolytic property in doses much larger than those used in the experiments.

*Experiment I (Thomsen's method)*

*Complement titrations*

COMPLEMENT (1-10)	0.55	0.6	0.65	0.7	0.75	0.8	0.85	0.9	0.95
Alcoholic antigen 1-10 { 2.0 cc.....		c	c	c	c	c	c	c	c
{ 3.0 cc.....							ac	c	c

One hour preliminary incubation. Addition of cells sensitized with two amboceptor units. One hour final incubation.

c = Complete laking.

ac = Almost complete laking.

	POSITIVE SERUM DILUTED AS FOLLOWS							
	1-2	1-4	1-6	1-8	1-10	1-12	1-16	1-20
Antigen, 2.0 cc. } .....	++++	++++	++++	+	0	0	0	0
Complement, 0.6 cc. }								
Antigen, 3.0 cc. } .....	++++	+	0	0	0	0	0	0
Complement, 0.9 cc. }								

++++ = Complete inhibition.

0 = No inhibition.

*Experiment II (Thomsen's method)*

*Complement titrations*

COMPLEMENT (1-10)	0.35	0.4	0.45	0.5	0.55	0.6	0.65	0.7
Antigen { 1.0 cc.....		c	c	c	c	c	c	c
{ 2.0 cc.....				c	c	c	c	c
{ 3.0 cc.....							c	0

		POSITIVE SERUM DILUTED					
		1-6	1-8	1-12	1-16	1-20	1-25
Antigen, 1.0 cc.	}	++++	++++	++++	++++	++++	++
Complement, 0.4 cc.							
Antigen, 2.0 cc.	}	++++	++++	++++	++++	+	+
Complement, 0.5 cc.							
Antigen, 3.0 cc.	}	++++	++++	++++	+?	0	0
Complement, 0.65 cc.							

*Experiment III (Thomas and Ivy's method)*

COMPLEMENT (1-10)		0.35	0.4	0.45	0.5	0.55	0.6	0.65	0.7
Antigen	1.0 cc.		c	c	c	c	c	c	c
	2.0 cc.				ac	c	c	c	c
	3.0 cc.						ac	c	c

Above complement titration done in presence of 0.2 pooled normal serum.

		POSITIVE SERUM DILUTED						
		1-6	1-8	1-10	1-12	1-16	1-20	1-25
Antigen, 1.0 cc.	}	++++	++++	++++	++++	++++	+++	+
Complement, 0.4 cc.								
Antigen, 2.0 cc.	}	++++	++++	++++	++++	+++	+	0
Complement, 0.55 cc.								
Antigen, 3.0 cc.	}	++++	++++	+++	+++	0	0	0
Complement, 0.64 cc.								

*Experiment IV (Thomas and Ivy's method)*

COMPLEMENT (1-10)		0.25	0.3	0.35	0.4	0.45	0.5	0.55	0.6
Antigen	0.5 cc.	ac	c	c	c	c	c	c	c
	1.0 cc.		c?	c	c	c	c	c	c
	2.0 cc.				c?	c	c	c	c
	3.0 cc.						ac	c	c

Titration done in the presence of 0.2 pooled normal serum.

		POSITIVE SERUM DILUTED						
		1-6	1-8	1-10	1-12	1-16	1-20	1-25
Antigen, 0.5 cc.	}	++++	++++	+++	++	+?	0	0
Complement, 0.32 cc.								
Antigen, 1.0 cc.	}	++++	++++	++++	++++	++	0?	0
Complement, 0.35 cc.								
Antigen, 2.0 cc.	}	++++	++++	++++	0?	0	0	0
Complement, 0.45 cc.								
Antigen, 3.0 cc.	}	++++	++++	+	0	0	0	0
Complement, 0.55 cc.								

From the above observations it is evident that with this particular alcoholic beef heart extract the largest dose tested (3 cc. of 1-10 dilution) would not so readily detect a weak positive serum as the next smaller dose (2 cc.), and this dose in turn would not give as good fixation with weak positive sera as 1 cc. On the other hand the still smaller dose does not give quite as good a result and therefore the optimal dose is approximately 1 cc.

In determining the most favorable dose of antigen therefore, one should ascertain the correct amount of complement for use with each of several doses of antigen, and then determine the complement-fixing power of each dose of antigen with its appropriate complement dose by testing it with a progressively diluted positive serum. *The dose of antigen which gives fixation with the highest dilution of positive serum is the optimal dose and this is not by any means usually the largest dose tested.*

The probable explanation of this observation is that when the dose of antigen is increased beyond a certain point the amount of complement which has to be added to overcome the anti-complementary effect is too great to be fixed by certain grades of positive serum.

The method described for ascertaining the dose of antigen is of course applicable only to those systems in which the complement dose is adjusted to the dose of antigen (Thomsen, Boas, Stern, Thomas and Ivy, Browning and McKenzie). In the other systems (Wassermann, Citron, Noguchi, Walker and Swift) in which the dose of complement is either fixed (0.1 cc.), or is two hemolytic units, the rule does not apply because the

ratio of complement to antigen is always greater and hence the more one can increase the antigen dose (and still remain below the point where non-specific fixations are obtained) the better the results.

# A STUDY OF TWO HUNDRED AND NINETY POST MORTEM WASSERMANN REACTIONS<sup>1</sup>

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The diagnostic value of the Wassermann test for syphilis performed on sera from human blood taken post mortem has been the subject of investigation and discussion for the last ten years. Practically all published reports on the work have been made in Germany. The first to carry out this research, Much and Fraenkel (1) and Pick and Proskauer (2), concluded that the Wassermann reaction gave reliable results when done on post mortem blood and that the results confirmed the prevailing comprehension of fibrous meso-arteritis, aneurism of the aorta, etc., as syphilitic lesions. Later investigators, on the contrary, arrived at different conclusions. Beside others, Krefting (3), de Besche (4) and Von Bruck (5) decided against the employment of the Wassermann reaction on post mortem blood because they found a very considerable number of positive reactions in cases where not the slightest probability of the existence of syphilis was apparent. Von Bruck expressed the notorious assertion that "the Wassermann reaction is a biological, not a cadaverish phenomenon." A succession of other investigators meanwhile took similar views, although less antagonistic. Seligmann and Blume (6), Nauwerck and Weichert (7), Lubarsch (8), Lucksch (9), Gruber (10), von Werdt (11), Schmidt (12), Reinhardt (13), Löhlein (14) and others found generally most positive post mortem reactions among syphilitics, but yet a number also among patients in whom presumably no syphilis had existed. The opinion prevailed that the positive Wassermann

<sup>1</sup> Read before the Mississippi Valley Medical Association at Indianapolis, October 11, 1916.

reaction on post mortem blood had only a relative value because such reactions now and then appeared among non-syphilitics, although most often among patients in whom syphilis was evident.

In 1913 Boas and Eiken (15), to whose excellent article on The Significance of the Wassermann Reaction Carried out on Cadaver Blood, we are indebted for much of the review of the German work, performed the test on sera from 540 bodies "in the usual way" with serum in decreasing amounts and with alcoholic extract of human heart as antigen. They reported 142 sera putrid or coagulated in inactivation and 25 anticomplementary, leaving 373. Using 0.2 cc. of human serum they found in brief the following:

- 33 Wassermanns positive: Syphilitic organ changes or syphilis in histories.
- 4 Wassermanns negative: Syphilitic organ changes.
- 9 Wassermanns negative: No syphilitic organ changes but syphilis in histories.
- 25 Wassermanns positive: No syphilitic organ changes and no syphilis in histories.
- 1 Wassermanns positive: Serum taken during narcosis; no syphilis.
- 301 Wassermanns negative: No syphilitic organ changes and no syphilis in histories.

It is worth while to recall that Boas and Eiken did not agree with Krefling that one will always find a positive Wassermann reaction on post mortem sera from patients with syphilitic lesions. One will not always so find the reaction among living patients. The phenomenon among 9 patients with negative post mortem Wassermann reactions but with histories of syphilis earlier in life agreed perfectly with that which one could find also among living patients, they argued. The finding of 25 positive sera in apparent non-syphilitics, however, could not be explained satisfactorily by attributing the positive reactions to unknown latent syphilis. Boas and Eiken found quite different results on the same sera when using 0.1 cc. instead of 0.2 cc. Under these conditions 22 of the 25 cases without evident syphilis gave a negative reaction instead of a positive, while only 7 of the 33 positive reactions among syphilitics became negative.

Candler and Mann (18) examined post mortem both blood and

cerebro-spinal fluid in 92 cases and cerebro-spinal fluid alone in 20 cases. Their technique was that of the original Wassermann. Their patients had been inmates of an insane asylum. The results they obtained confirmed clinical and anatomic diagnoses in 110 of 112 examinations of cerebro-spinal fluid and in 86 of 92 examinations of blood. Without going into further discussion of their work their conclusions are noted as follows:

While, therefore, our observations lead us to consider that the reactions on the blood and cerebro-spinal fluid removed from the cadaver before decomposition has commenced will give reliable results, we agree with Luksch that decomposition is liable to alter the reaction, as may also a terminal microbial infection. This change, however, is not confined to the alteration of a negative to a positive result, but may alter a positive into a negative; and we would point out that decomposition may similarly influence the result of the test on fluids and serums removed during life.

To ensure satisfactory results on post mortem material it is, therefore, necessary to remove the cerebro-spinal fluid and blood as soon as possible after death. The serum should be separated and inactivated at once, and the cerebro-spinal fluid should be carbolyzed, unless the test can be applied the same day. The same remarks apply with regard to the preservation of material removed during life.

The only American publication we can find on this subject was made by Moody, Jackson and LeCount (19). Two years ago they examined 102 cerebro-spinal fluids and 88 blood sera from dead bodies. They used the Wassermann-Noguchi (human-rabbit) serum test in about four-fifths of their cases, the chicken-rabbit system in one-fifth and Lange's colloidal gold test in 57 spinal fluids. Their results were briefly as follows:

<i>Positive</i>			
	WITH ANATOMICAL EVIDENCES OF LUES	WITH NO ANATOMICAL EVIDENCES OF LUES	TOTAL
Cerebro-spinal fluid.....	41	15	56
Blood.....	57	9	66
<i>Negative</i>			
Cerebro-spinal fluid.....	27	19	46
Blood.....	10	12	22

The 24 positive reactions obtained with fluids from bodies with no gross evidences of syphilis, equivalent to 2.8 per cent of the 190 examinations, concerned deaths from the following: Delirium tremens and broncho-pneumonia, 5; cerebral hemorrhage, 4; uremia, 3; tumor, 3; ascending infection of urinary tract, 2; cirrhosis of liver with icterus, 2; peritonitis, 1; miliary tuberculosis, 1; diabetic coma, 1; chronic heart disease, 2 (total 24).

Their conclusions were:

It would seem fair to state that under certain conditions the Wassermann reaction in the fluids collected after death is of value. Where the blood or spinal fluids have remained in the dead bodies long enough for post mortem changes to occur in them, then the results cannot be relied on. Finally, in individuals dying from acute infections the test is often positive in many instances independently of a syphilitic infection or its absence.

The Lange test we regard of great service to differentiate syphilis from other lesions of the cerebro-spinal system in those cases where a clear spinal fluid uncontaminated with blood is obtained; otherwise it is valueless.

Before coming to our own results we should like to recall that Luksch (9) in 1910 stated that a positive Wassermann reaction shortly before or after death, in his opinion, did not have much value because the changes in blood serum produced by serious disease caused the phenomenon of fixation or deviation of complement as in syphilis. He cited a case of a man who had had positive clinical syphilis, had infected his wife, the latter giving birth to a son with congenital syphilis, and who, although he had had a negative reaction during life, became septic and died, giving a positive reaction after death. Ergo: the post mortem Wassermann was not reliable. Taking for granted that such reasoning is sound, it may be argued conversely that if a patient who is not septic gives a positive post mortem Wassermann, the reaction may be taken as evidence of syphilis. Lubarsch, in discussing the work of Luksch, made a plea for uniform technique. In the earlier days of the application of the Wassermann reaction positive results were found by



many different workers in many non-syphilitic diseases. In this connection the observation of a recent writer (17), to the effect that there have been almost as many different methods of performing the Wassermann test as there are stars in heaven, contained more truth than facetiousness.

The tests discussed in the remainder of this paper have been done on sera taken under aseptic precautions from 290 patients who have come to autopsy in the pathological laboratory of the Medical Department of the University of Louisville and of the Louisville City Hospital. In taking the blood the right auricle, as a rule, has been seared with a hot iron and stabbed with a flamed scalpel. The blood has been sucked up into a sterile pipette plugged with sterile cotton in its proximal end and allowed to flow back down into a sterile test tube and then stored in an ice box until used. Tests have been made twice a week so that no serum has been kept at the longest more than eighty-four hours and then under cold, sterile conditions. The serum has rarely stood in contact with the clot in the tube longer than forty-eight hours. The bodies have been those of patients that have died in the City Hospital and of patients that have died outside the hospital and have been sent to the laboratory by the coroner for examination. The tests have formed a part of about 4000 Wassermanns done in the laboratory during the last two years. In every case the serum has been numbered and the serologist has not known the origin of the serum at the time of performing the tests. The Wassermann sheep-rabbit hemolytic system and at least two antigens have been employed in each test, the antigens including at different times preparations of alcoholic extract of human or beef or guinea pig heart cholesterinized and the acetone-insoluble fraction of alcoholic extract of beef heart. Amboceptor and complement have been titrated immediately before use in each test. The dose of human serum has been uniformly 0.1 cc., the amount with which Boas and Eiken claimed to have secured most reliable results. All tests have been individually controlled and each set collectively controlled with known positive and negative sera. Inhibition has been indicated + for 25 per cent inhibition, ++ for 50 per cent,

+++ for 75 per cent and ++++ for full positive or 100 per cent lack of hemolysis. The greatest number of hours post mortem after which a serum gave a positive reaction was 24, while the greatest number after which a serum gave a negative reaction was 30. The former was from a male negro who showed a typical saddleback nose, chronic aortitis, erosions of the entire middle portion of the superior maxilla and hard palate; the latter from a patient who showed lobar pneumonia and no signs of syphilis. The anticomplementary sera were from 6 to 12 hours post mortem.

The results can be best shown in tabulated form.

TABLE I

290 cases

Class I	76	Wassermanns positive with apparent syphilitic anatomical lesions or positive evidence of syphilis in histories.
Class II	161	Wassermanns negative with no apparent syphilitic anatomical lesions and no evidence of syphilis in histories.
Class III	8	Anticomplementary.
Class IV	7	Wassermanns negative with apparent syphilitic anatomical lesions or positive evidence of syphilis in histories.
Class V	38	Wassermanns positive with no apparent syphilitic anatomical lesions and no evidence of syphilis in histories.

In looking over these results we are struck in the first place with the fact that we find *no* sera putrid or coagulated, whereas Boas and Eiken report 142 in 540 so damaged. This bespeaks confidence in the condition of the sera in general. This confidence is strengthened by finding only 8 sera, or less than 3 per cent, anticomplementary. In Louisville the summer heat is terrific, the writer having seen the mercury stand at 114° F. on the shady side of the federal obelisk in the Postoffice Square. Under such conditions more anticomplementary sera might have been expected. Confidence in the general results is again strengthened by finding expected reactions in classes I and II, including 237 sera of 282 (excluding anticomplementary sera). In other words in slightly more than 84 per cent of the cases the result of the post mortem Wassermann test confirmed the apparent clinical conditions. The fact that 7 of 282 sera failed to show positive reactions in cases which revealed at autopsy anatomical

lesions regarded as syphilitic cannot be used as evidence against the reliability of the post mortem Wassermann done under proper conditions because no better results could be expected ante mortem. It is interesting to note that the autopsy in each of these 7 cases revealed an aneurism and that 6 of the 7 patients were negroes, 2 male and 4 female. In the only case in which an ante mortem Wassermann had been done the ante mortem and post mortem results agreed. The 7 cases were as follows:

TABLE 2

*Wassermanns negative; aneurisms found post mortem*

1. (15-10) Ruptured aortic aneurism; negro; female; outside coroner's case; head restricted; no opportunity to take history.
2. (15-12) Aneurism of aortic arch; negro; female; 32 years old; died a few minutes after admission to hospital; coroner's case; no opportunity to take history.
3. (15- 13) Ruptured aortic aneurism; negro; female; outside coroner's case; no opportunity to take history; head restricted.
4. (15- 26) Aneurism of aorta; negro; male; patient in hospital one month; says he had syphilis 25 years ago; head restricted.
5. (15-143) Aneurism of arch; white; female; age 63; patient on psychopathic ward six weeks with diagnosis of paralysis agitans. Head restricted. Antemortem Wassermann negative.
6. (16- 71) Aneurism of aorta; negro; female; coroner's case outside; no history obtainable.
7. (16-113) Ruptured aneurism of aorta; negro; male; coroner's case outside; no history obtainable.

So far, then, we have found everything to indicate the reliability of the post mortem Wassermann done under proper conditions. We must now direct attention to class V, embracing positive Wassermann reactions in cases which showed no anatomical lesions which could be regarded as syphilitic and which offer no positive evidence of syphilis in their histories. To study these fairly one must separate them into three subdivisions as follows:

TABLE 3

*Class V. Wassermanns positive. No syphilis evident*

A. Bodies sent by coroner to laboratory for autopsy. No history whatever Examination often partially restricted to evident cause of death.	
Stabbed to death (Wassermann + and Wassermann ++ in two cases).	3
Cardio-renal (Wassermann + in one case).....	3
Shot to death.....	2
Lobar pneumonia.....	2
Burned to death.....	1
Choked to death.....	1
Skull fractured.....	1
Generalized tuberculosis.....	1
Total.....	14
B. Patients admitted to hospital but coming to autopsy as coroner's cases because dying within 24 hours or without obvious cause. Little or no opportunity for history or physical examination.	
Lobar pneumonia.....	2
Pulmonary tuberculosis.....	1
Generalized tuberculosis (Wassermann only +).....	1
False elephantiasis of genitals.....	1
Burned to death.....	1
Stabbed to death.....	1
Traumatic septicemia.....	1
Total.....	8
C. Patients admitted to hospital. Autopsy permission from next of kin or friends or municipal authorities in cases of patients without relatives or friends.	
Generalized tuberculosis.....	3
Lobar pneumonia (one only +).....	2
Cardio-renal (one only +).....	2
Acute appendicitis and peritonitis (only +).....	1
Streptococcus meningitis (only ++ ).....	1
Gangrene of intestine, intestinal obstruction.....	1
Acute pyelonephritis.....	1
Acute tubular nephritis (Negro, male, age 20).....	1
Shot to death.....	1
Carcinoma.....	1
Pulmonary tuberculosis.....	1
Lobular pneumonia (Negro baby).....	1
Total.....	16

Before considering these cases more carefully any one who has worked in a large, public, southern hospital will be struck at once with the significance of the statistics in the following table.

TABLE 4

*Showing race and sex of cases with positive Wassermanns and no apparent syphilis*

A. (C. O.)	14 cases	Negro	{ Male, 7 Female, 6 }	13	
		White	{ Male, 1 Female, 0 }		
B. (C. H.)	8 cases	Negro	{ Male, 3 Female, 3 }	6	Total negro males, 17 Total negro females, 16 Total white males, 5 Total white females, 0
		White	{ Male, 2 Female, 0 }		
C. (H.)	16 cases	Negro	{ Male, 7 Female, 7 }	14	
		White	{ Male, 2 Female, 0 }		

There are two methods of studying any question. One is to scrutinize all small details. The other is to stand back and view it in its entirety with broad perspective. Let us consider these 38 cases, for they are the only ones in this series which could possibly offer any evidence against the value of the post mortem Wassermann, first from the first viewpoint. We may immediately eliminate 6 because they showed only a one + reaction, or not more than 25 per cent inhibition, which can hardly be construed as a specific reaction in the absence of any clinical evidence of syphilis. This leaves 32. Of these, 22 offered no opportunity whatever for history and practically none for physical examination and in many a complete autopsy was not permitted. These patients may have been suffering from unknown, latent syphilis or have been under treatment. There remains 10 cases admitted to the hospital and coming to autopsy in the ordinary way. Of these 3 showed only a "one +" positive reaction, leaving 7 in which at least 50 per cent inhibition indi-

ected luetic infection. Should a skilled clinician working in the wards of a southern public hospital, be satisfied if he could eliminate ante mortem, to this extent; 7 cases in 290 in which he could not establish objective syphilis although those 7 patients had positive Wassermann reactions?

Now let us step back and consider these cases with broad perspective. In the first place, taking for granted that an unquestionably skillful and persistent effort is always made in a public hospital to obtain a perfect history, recollect that 87 per cent of the patients in this class were negroes, almost equally divided as to sex, and that many of them came from the lowest and most unfortunate walks of life. The prevalence of syphilis among negroes is notorious. Their stories in hospital wards are notoriously unreliable. The average negro may not mean to lie; he simply does not know how to tell the truth—and if he is truthful he may not know that he has had syphilis.

It has been argued that, because the Wassermann appears positive in cases in which syphilis is not objective nor historic while various infectious diseases are obvious, that these diseases must bring about changes in the blood which cause complement fixation. Would it not be more logical to conclude that deaths in these cases have resulted from the most common fatal diseases, such as pneumonia and tuberculosis, to say nothing of violence, in patients who were mostly ignorant or unfortunate or vicious colored people and who were simultaneously victims of early or late or treated syphilis? If the positive Wassermann is to be attributed to pneumonia or tuberculosis or other infectious lesions or to malignant tumors because they appeared in patients who had died of those lesions, of what weight is that argument when it is shown that, of the 161 cases in this series which gave negative post mortem Wassermans and presented no evidence of syphilis, 94 were negative in patients dying from the following diseases: Lobar pneumonia, 24; pulmonary tuberculosis, 16; lobular pneumonia, 11; acute peritonitis, 11; acute nephritis, 6; carcinoma, 5; acute vegetative endocarditis, 4; generalized tuberculosis, 4; tuberculous entero-colitis, 2; pneumococcus meningitis, 2; streptococcus meningitis, 1; meningo-

coccus meningitis, 1; empyema, 1; perihepatic abscess following gastric ulcer, 1; acute pericarditis, 1; brain tumor, (not yet diagnosed), 1; hypernephroma, 1; lymphosarcoma, 1; and carcinoma with infectious nephritis and aneurism of the aorta, 1? The answer may be applied to class V in table 3 above.

Boas and Eiken (15) checked ante mortem Wassermanns against post mortem Wassermanns. Their results did not confirm the observations of some earlier investigators that a positive ante mortem reaction occasionally changed to a negative post mortem reaction or vice versa, except in a single case. In 11 control cases the test shortly ante mortem and post mortem were consistently negative. In 7 control cases ante mortem and post mortem tests showed so little difference in the strength of the positive reactions that the difference was considered by the authors to come within the bounds of experimental error. In 1 case of gumma of the kidney they found a positive reaction before death change to negative after death, a confirming percentage of 95.

In our series there have been 37 control cases. During the early part of the investigation it was not possible to secure many ante mortem Wassermanns. They are increasing, however, and we purpose to report comparisons in a larger series. Our control cases are shown in the following table:

TABLE 5

*Comparison of ante mortem and post mortem reactions*

Ante mortem positive followed by post mortem positive .....	16
Ante mortem negative followed by post mortem negative.....	17
Ante mortem positive followed by post mortem negative.....	2*
Ante mortem negative followed by post mortem positive.....	2*

\* See below.

It behooves us to study the apparent discrepancies in the last 4 cases. Of the cases giving positive post mortem reactions following negative ante mortem reactions 1 was in a negro female (16-86) 45 years old who had been in the hospital 6 days. She had been a morphine habitue for 20 years, but denied syphilis. The blood for the ante mortem Wassermann had been taken the day before death. The head was restricted. The woman had

died of pneumonia and vegetative endocarditis. The post mortem reaction with human heart cholesterinized antigen was read positive +; with beef heart cholesterinized antigen =. Such close reactions in the face of negative anatomical findings and in the light of a morphine history for 20 years are not worth considering. The other case was that of a negro male 49 years old, who had died of generalized tuberculosis. There were no lesions that might be considered remotely syphilitic. The history does not mention the question of syphilis. The ante mortem negative Wassermann was taken ten days before death. Here is a flat discrepancy (16-178) which is not explained. Of the 2 cases giving negative post mortem Wassermanns following positive ante mortem Wassermanns 1 was in a female negro (15-202) about 50 years old who died of lobar pneumonia. She had been paralyzed several weeks. The autopsy showed hemorrhage into the internal capsule and lenticular nucleus with marked beaded sclerosis of the basilar arteries of the brain. The ante mortem Wassermann had been done about seven weeks before death and had shown the slightest degree of inhibition, being recorded as doubtfully positive. A discrepancy between a doubtfully positive ante mortem reaction and negative post mortem reaction is another discrepancy not worth considering. The remaining case in this class is that of a male negro who died of oedema and chronic passive congestion of the lungs. He had been in the hospital only three days and the ante mortem Wassermann had been positive + + +. There was no history. Of the 37 cases, then, in which we have an ante mortem Wassermann with which to check the post mortem Wassermann there are two flat discrepancies which are not explained. This is also a confirming percentage of 95. Any series of complement fixation reactions which offers corroboration in repeated tests in 95 per cent of cases under constantly changing conditions of patients may be regarded as reliable. The greatest number of hours post mortem after which a negative confirmed an ante mortem negative was 22; positive, 6.

Finally it may be figured that 114 of 282 sera which gave reactions were positive (only 8 not more than "one +"), or



40.4 per cent. The results of previous investigations tabulated by Moody, Jackson and LeCount (19), with theirs and ours, compare as follows:

TABLE 6  
*Comparison of results in different series*

OBSERVERS	NUMBER OF CASES	POSITIVE	PER CENT
Bruck.....	101	59	58.4
Seligman and Blume.....	100	28	28.0
Schlimpert.....	361	46	18.7
Luksch.....	309	145	46.0
Simmonds.....	160	33	21.0
Lubarsch.....	262	55	21.0
Löhlein.....	149	42	28.2
Veszpremi.....	100	46	46.0
Krefting.....	115	43	37.0
Nauwerck and Weichert.....	206	57	28.0
Grüber.....	300	101	30.0
Moody, Jackson and LeCount.....	88	66	75.0
Graves.....	282	114	40.4
Total.....	2533	835	32.9

The positive sera in our series were distributed according to race and sex as follows:

TABLE 7  
*Distribution of positive sera*

	MALE	PER CENT	FEMALE	PER CENT	TOTAL	PER CENT
Negroes.....	55	48.2	33	28.9	88	77.1
White.....	21	18.4	5	4.3	26	22.7
Total.....	76	66.6	38	33.2	114	99.8

Of what use is a post mortem Wassermann reaction? Not infrequently one surveys a lesion at autopsy and cannot venture to decide whether it had its origin in syphilis or not. Not always will the microscope furnish deciding assistance even with such fine technique as that published recently by Warthin. Many times no reliable history is obtainable. It is impossible to investigate as to the existence of unknown, latent syphilis or a fresh infection. If the post mortem Wassermann reaction may be relied

upon it affords material help in diagnosis of pathologic anatomy. When the Wassermann reaction test is carried out as a routine on all patients in the wards of a great hospital one finds many apparently non-specific, positive reactions. However, if one searches deeper into the histories of these patients or observes them for a time they practically always prove to be syphilitics, either confessing to a very old or fresh infection or later exhibiting syphilitic symptoms. At autopsy such investigation is impossible. In this series 76 of 84 patients with apparent syphilitic anatomic lesions or with positive evidence of syphilis in their histories gave a positive reaction; a per cent of 90.4. This is about as high as the percentage of positive reactions quoted by many authors for living patients with acute lues.

Before drawing conclusions a word must be said about the specificity of the Wassermann reaction. Previous mention has been made of the widely differing results secured by earlier workers. The Wassermann is a delicate biochemical test. No diagnostic method in the history of medicine has been more grossly abused or more dishonestly exploited. In the hands of a competent serologist the Wassermann is the most constant and delicate single symptom of syphilis, according to Kolmer (20), who believes it is practically always specific for syphilis except in yaws, which may be a form of syphilis, and leprosy. Boas did not find a single negative in the examination of 437 sera from untreated cases of secondary syphilis. Kolmer's experience has been the same.

The last German work reported is that of Boas and Eiken (15) who conclude:

In the study of cadaver blood with 0.1 cc. of serum (half the accustomed dose) the reaction is approximately specific because, of 326 control patients, only 3 gave positive Wassermann reactions. On the contrary the negative Wassermann reaction, performed in this way on cadaver blood, has as little significance as the negative Wassermann reaction on living patients because, among 29 untreated active cases of syphilis, not less than five times the negative Wassermann reaction appeared.

## CONCLUSIONS

1. Post mortem Wassermann reactions confirmed ante mortem reactions in 95 per cent of 38 control cases. Positives were confirmed in serum six hours post mortem and negatives in serum twenty-two hours post mortem.

2. In 90.4 per cent of cases showing post mortem anatomic lesions of syphilis or presenting positive evidence of syphilis in their histories the sera post mortem gave positive Wassermann reactions.

3. The fact that positive post mortem reactions appeared in 38 cases, which did not present post mortem lesions or historic evidence of syphilis and in which death was due to acute infections, tuberculosis or malignant tumors, cannot be interpreted to mean that the reaction was caused by those diseases because, in the first place, the histories and autopsies in those cases were not nearly enough complete to rule out syphilis, and in the second place, because sera from 94 patients who died of acute infections, tuberculosis or malignant tumors, examined under similar conditions, gave negative reactions.

4. Only 7 per cent of 282 cases showed negative reactions in the presence of anatomical lesions (aneurisms) characteristic of syphilis.

5. The reactions conformed to the anatomic and historic evidence in 84 per cent of the cases.

6. The fact that only eight, or 3 per cent, of the sera were anticomplementary indicates that the sera were in good condition.

7. The average percentage of specific reactions was almost as high post mortem as would be expected ante mortem.

8. The positive reaction appeared in twice as many males as females, in three times as many negroes as whites and in white females in only 4.3 per cent of the cases examined.

9. The Wassermann reactions, performed on post mortem blood according to the methods followed in this investigation, is a reliable aid to the diagnosis of syphilis.

In conclusion I wish to express my thanks for the coöperation in this investigation by members of the laboratory staff, Drs. J. W. Moore, H. H. Reeder, H. R. Livesay and T. R. Maxwell and Mr. E. E. Butler.

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## A STUDY OF VON DUNGERN'S INDIGO TEST FOR SYPHILIS

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In the issue of the Muenchener Medizinische Wochenschrift or September 7, 1915 (No. 36), von Dungern described a test for the serum diagnosis of syphilis with chemical reagents. Although an adequate theoretical basis for this phenomenon had not as yet been secured, von Dungern believed that it was practically applicable to the diagnosis of syphilis.

The test was founded upon the observation of Karl Jaudas, von Dungern's laborant, that the heat coagulation of normal serum is prevented by a smaller relative quantity of an alkaline solution of indigo than is the case with the sera of syphilitics.

He prepared the indigo reagent in the following manner:

One gram of indigo is dissolved in 4 cc. of concentrated sulphuric acid and distilled water is added to make 100 cc.; 15 cc. of this solution are diluted with 10 cc. of water; to four parts of this dilution one part of Fehling's solution No. 2 is added. The resulting solution should be greenish yellow in color and free of all particles. It must be used within fifteen minutes of its preparation.

The serum to be tested is inactivated by heating it at 54° C. for one-half hour. To 0.3 cc. of the inactivated serum there is added 0.2 cc. of the freshly prepared reagent. The tubes after standing at room temperature for one-half hour are immersed in boiling water for exactly one minute. Readings are made after the tubes have stood for two hours longer at room temperature. The tubes must be thin walled and of narrow caliber so that the heat is readily conducted to the serum mixture. Positive sera should show at the end of two hours a gelatinous coagulation: negative sera remain fluid.

Von Dungern refers the greater coagulability of syphilitic sera to a higher serum albumen content, which he thought he

could demonstrate by means of the immersion refractometer. He states that a non-specific coagulation of normal sera may be obtained by the process if a smaller amount of the indigo reagent is used.

Von Dungern examined 250 sera, which were also tested with the Wassermann reaction. A number of these sera were from undoubtedly syphilitic individuals. Most of the Wassermann positive sera reacted positively with the coagulation test. In several cases of undoubted syphilis the sera were negative with the Wassermann test but reacted positively with the coagulation test and several non-syphilitic but Wassermann positive sera were negative with the coagulation test. On the other hand, a positive coagulation reaction was obtained with several non-syphilitic sera; viz: six cases of bone tuberculosis, one carcinoma of the larynx and two cases of gout.

Having at my disposal ample clinical material and a specimen of indigo, obtained through Dr. Coca from Laborant Jaudas in von Dungern's laboratory, I was able to undertake a further study of this reaction. Although the technique of the test as described was apparently simple, our first difficulty was met in the preparation of the reagent. It was impossible to dissolve the prescribed amount of indigo (1 gram.) in either 4 cc. or 7 cc. of cold concentrated sulphuric acid. Neither trituration nor standing for twenty-four hours resulted in solution. However it was found<sup>1</sup> that a solution could be obtained by heating the mixture over a low flame for a few minutes and then rapidly adding water. A solution was thus obtained with 0.5 gram of indigo and 2 cc. of concentrated sulphuric acid. Distilled water was added to 50 cc. and the solution was centrifuged. A very small amount of sediment was thrown down and the clear supernatant fluid was kept as a stock solution. The Fehling solution No. 2 was prepared as directed.

17.3 grams of Rochelle salt were dissolved in 34 cc. of 15 per cent sodium hydroxide, and water was added to make 50 cc. The final mixture was prepared as directed. 1.5 cc. of the indigo sulphuric acid were diluted with 10 cc. of water and to 4 cc. of

<sup>1</sup> I wish to thank my friend Mr. J. Boek for assistance at this point.

this solution there was added 1 cc. of Fehling's solution No. 2. The addition must be made rapidly and with a blow-out pipette, otherwise a precipitate is formed which may not redissolve for five to ten minutes. This precipitate sometimes forms in spite of every care. The final solution was a greenish yellow color but this gradually changed to a brown. As advised by von Dungern, the reagent was always used within fifteen minutes after the final mixture was prepared.

Calculation of the chemical composition of these various mixtures shows that the final mixture is alkaline, one cubic centimeter of it requiring 1.21 cc. of  $\frac{N}{10}$  acid for its neutralization; that it contained 1.1 per cent of sodium sulphate and 6.9 per cent of sodium and potassium tartrate.

The preliminary tests were performed with various amounts of the reagent in order to determine at just what point coagulation is obtained with normal sera. The amounts of reagent used were 0.2 cc., 0.1 cc., 0.05 cc. and 0.025 cc., these being added to 0.3 cc. portions of the inactivated sera and the mixtures being then treated as described by von Dungern. It was found that those sera which coagulated showed a superficial zone of blue color and a loss of color in the deeper portions. Those that did not coagulate remained a yellowish brown. The results of these tests with normal (Wassermann Negative) sera are shown in table 1.

TABLE 1  
*Normal sera (Wassermann negative)*

0.3 CC. INACTIVATED SERUM	COAGULATION	SEMI COAGULATION	NO COAGULATION
0.2 cc. reagent.....	0	3	93
0.1 cc. reagent.....	37	5	50
0.05 cc. reagent.....	23	0	5
0.025 cc. reagent.....	9	0	0

It was necessary to treat but a few sera with a quantity of reagent less than that recommended by von Dungern for it was soon learned that nearly all normal sera coagulated with these quantities viz., 0.05 cc. and 0.025 cc. With 0.1 cc. of the reagent 40 per cent of the sera coagulated and with 0.2 cc. all of them remained fluid, or semi-fluid.

In a like manner a series of tests were carried out on the sera of untreated syphilitics. Most of these cases were seen by myself and all showed active lesions of the primary and secondary stages of the disease. Wassermann tests were not performed in these cases. The results of these tests are shown in table 2.

TABLE 2  
*Sera of untreated syphilitics*

0.3 CC. INACTIVATED SERUM	COAGULATION	SEMI COAGULATION	NO COAGULATION
0.2 cc. reagent.....	2	0	22
0.1 cc. reagent.....	8	4	9

We were able to obtain sera from fifteen additional cases in which the Wassermann test resulted positively. None of these cases was seen by myself. The results are shown in table 3.

TABLE 3  
*Wassermann positive sera*

0.3 CC. INACTIVATED SERUM	COAGULATION	SEMI COAGULATION	NO COAGULATION
0.2 cc. reagent.....	1	0	14
0.1 cc. reagent.....	6	2	6
0.05 cc. reagent.....	3	0	0

Comparing the results of the coagulation test in the normal and the syphilitic series, we see that where 0.2 cc. of the indigo reagent was used, there was only a slight difference in the average behavior of the sera; i.e., the difference between three semi-coagulations and three complete coagulations. Where 0.1 cc. of the reagent was used the difference was also inconsiderable, 57 per cent of the syphilitic series showing one coagulation as against 45 per cent of the non-syphilitic series.

These differences are obviously unavailable as a basis for a clinical diagnostic test.

In order to determine whether the indigo or the salts in the reagent play any part in the reaction, we prepared mixtures in which one or both of these ingredients were absent, or present in only small amount. Four cubic centimeters of concentrated sulphuric acid were diluted to 100 cc. with water: 1.5 cc. of this



were further diluted with 10 cc. of water. To four parts of this final dilution one part of Fehling's solution No. 2 was added. This gave a mixture identical with that of von Dungern, but without the indigo. Sodium hydroxide was dissolved in 0.8 per cent sodium chloride in such concentration that its alkalinity was the same as that of von Dungern's final mixture; i.e., 1 cc. = 1.21 cc.  $\frac{N}{10}$  alkali. This solution represented the reagent of von Dungern minus both the indigo and the high salt concentration. Various amounts of these two solutions and of von Dungern's reagent were added to 0.3 cc. portions of inactivated sera and these mixtures were treated as usual. The results of these comparative tests carried out with five different normal (i.e., Wassermann negative) sera are shown in table 4.

TABLE 4

*Comparative anti-coagulating action of von Dungern's reagent and of the reagent minus the indigo and minus the indigo and the salts*

	0.3 CC. INACTIVATED SERUM WITH	0.2 CC. REAGENT	0.1 CC. REAGENT	0.5 CC. REAGENT	0.25 CC. REAGENT
1.....	a	0	0	0	0
	b	0	0	0	0
	c	0	0	0	+—
2.....	a	0	0	+	+
	b	0	0	+	+
	c	0	0	+	+
3.....	a	0	+	+	+
	b	0	+	+	+
	c*	0	0	0	0
4.....	a	0	0	+	+
	b	0	0	+	+
	c	0	0	+	+
5.....	a	0	+	+	+
	b	0	+	+	+
	c	0	+	+	+

a, Indigo-Fehling reagent; b, reagent without indigo; c, sodium hydroxide in 0.8 per cent sodium chloride; 0, no coagulation; +, coagulation; +— semi-coagulation.

\* This result may have been due to a technical error; the test was not repeated.

It is seen from the above table that apparently neither the indigo nor the salts play any part in the inhibition of heat coagulation by von Dungern's reagent. It is the alkali content of the reagent alone which exercises that effect.

#### SUMMARY

1. Von Dungern described an alkaline indigo solution that when added to an inactivated serum would inhibit its normal coagulation by heat. He believed that the indigo present was the active inhibiting substance. We have shown that neither the indigo nor the salt content of the reagent takes part in the inhibition of the coagulation. The alkali is the sole inhibiting agent.

2. Von Dungern further stated that with similar amounts of the reagent this inhibition was less marked in syphilitic than in normal sera. In our experience such a difference was present in but very slight degree and with no constancy.

3. The method is of no value in the serum diagnosis of syphilis for in our entire series of 140 sera, 40 of which were evidently syphilitic, coagulation occurred in but 3, with the prescribed amount of reagent.

# A COMPARATIVE STUDY OF DIFFERENT METHODS OF PERFORMING THE WASSERMANN TEST FOR SYPHILIS

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The Wassermann complement-fixation test for syphilis was at first regarded as an immunity test depending upon a reaction between antibodies specific for *Spirochaeta pallida*, on the one hand, and the specific protein constituents of this pathogenic organism, on the other hand. This conception had to be given up several years ago and at present it is quite generally believed that the test is not a specific one in the usual immunologic sense, but that it is a somewhat analogous reaction, which finds its justification in its practical value attested by experience, rather than in logical theoretical considerations. As a necessary corollary it results that a proposed antigen or a proposed technic finds favor not according to its theoretical specificity, but only through extensive and satisfactory use upon a large series of patients. Although clinical satisfaction, especially in a short series of cases, cannot, without reserve, be accepted as final proof of scientific accuracy, yet, in the long run, it is a very safe criterion.

A modification of the Wassermann antigen, which has gained very enthusiastic and almost general adoption, is that involved in the addition of cholesterin to the tissue extracts formerly used. In 1910, Browning, Cruikshank and McKenzie (1) showed that the quantity of complement absorbed by a mixture of lecithin (watery emulsion of an alcoholic solution of lecithin) and inactivated syphilitic serum could be greatly increased by the admixture of cholesterin. Gilmour (2) in 1911, maintained that the use of cholesterinized antigen furnished the most deli-

cate and most reliable method for the serum diagnosis of syphilis. Browning, Cruikshank and Gilmour (3) found lecithin derived from liver or heart to be more useful than that derived from egg-yolk or brain. Browning and Cruikshank (4) investigated a number of cholesterin derivatives, which they tested in combination with lecithin as antigens for the Wassermann reaction, but they found that none of these derivatives is as useful as pure cholesterin itself. Sachs (5) found that alcoholic extracts of normal tissues, when combined with cholesterin, possess antigenic properties equal to those of the extracts of syphilitic organs.<sup>1</sup> This view was confirmed by McIntosh and Fildes (6), who further concluded that in this combination the requirements of a standard antigen were finally fulfilled. Walker and Swift (7) ascertained by comparative tests that the most favorable concentration of cholesterin is 0.4 per cent added to the alcoholic extract of human heart muscle or guinea-pig heart.

There can be no question that the addition of cholesterin to the alcoholic extract renders the latter more powerful as an antigen and that considerably more positive results are thus obtained in the practical testing of human sera. The accuracy and reliability of these additional positive results have, however, been questioned. Thus Kolmer (8) pointed out that cholesterinized alcoholic extracts may give some inhibition of hemolysis with normal sera and must, therefore, be very carefully controlled. However, he places them first in order of practical efficiency in diagnosis. Zinsser (9) also has sounded a warning against too implicit reliance upon the cholesterinized antigen, as follows:

From the experience of many investigators it now seems unquestionable that additions of cholesterin increase the delicacy of the reaction in that more cases react positively with such an antigen than with the uncholesterinized preparations. The experience of Hopkins and Zimmermann, however, would indicate that great caution must be

<sup>1</sup> "Es scheint dass die Differenz, welche zwischen Extrakten aus normalen und syphilitischen Organen in ihrer Brauchbarkeit fuer die Wassermann'sche Syphilis-reaktion besteht, durch die Cholesterierung der normalen Extrakte aufgehoben wird."

exercised when the reaction is done in this way, since occasional positive results are obtained with cases clinically not syphilitic. These workers believe that cholesterinized antigen is extremely useful, but advise its use only parallel with the ordinary lipoidal antigens and together with careful study of the clinical aspects of the case.

McClure and Lott (10) have also concluded that cholesterin additions render the alcoholic extracts more sensitive but also somewhat less specific and that they are, therefore, of questionable value in the practical diagnosis of syphilis. Snow and Cooper (11) have reported partial to complete complement fixation with cholesterinized antigen in 31 per cent of sera from non-syphilitic tuberculous patients, although they obtained reliable results with the simple alcoholic extracts.

In practical work one is compelled, therefore, to perform the test with both kinds of antigen and to regard as somewhat doubtful those sera which give positive fixation with the cholesterinized antigen and not with the alcoholic extract. With the progress in diagnosis and treatment of syphilis and the more frequent appeal to the Wassermann test to aid in deciding about sufficiency of treatment, existence of latent syphilis or about the nature of low-grade pathological changes of slow evolution and slight extent, the proportion of instances in which there is definite disagreement between the results given by the two kinds of antigen has shown a tendency to increase. Frequently, in a case where the result of the Wassermann test is awaited with the greatest interest by the clinician, the serologist is unable to be of much assistance, because of this fact. We are convinced that the simple alcoholic antigens fail to detect syphilis in many instances of this kind in which a syphilitic lesion is actually present, but we are equally convinced that the cholesterinized antigen sometimes gives positive fixation in non-syphilitic patients suffering from other diseases, such as acute articular rheumatism, pneumonia, jaundice or tuberculosis. The appended tabulations show this very clearly.

Another matter of considerable importance in the Wassermann test is the temperature and the length of time employed for the first part of the reaction, the time during which takes

place the union between antigen and serum antibody and the binding or destruction of complement. Wassermann and most students of the subject have allowed this part of the reaction to take place at 37°C. Against this common practice may be urged the well recognized fact that complement spontaneously loses its power very much more rapidly at 37°C. than at 8° or 10°C. and the further well-known fact that antigen and specific immune body unite readily even at a temperature of 0°C.

McNeil (12) has presented the results of comparative tests upon 466 persons, most of whom were presumably syphilitic, employing for each case an incubation at 37°C. in one test and an incubation at ice-box temperature in the other. The antigen was the crude alcoholic extract. At 37°C. he obtained 176 positive reactions, 273 negative and 17 doubtful. At the ice-box temperature he obtained 224 positive reactions, 212 negative and 30 doubtful. This amounted to an increase in positive results of 10.3 per cent. Coca and L'Esperance (13) have also obtained positive fixation by the ice-box method with luetic sera which gave negative results in the incubator. They also report instances in which a syphilitic serum failed to give positive fixation in the ice-box while giving positive fixation at 37°C.

In this communication we wish to present the results of 496 Wassermann tests upon 477 patients by three different methods, together with brief clinical notes upon those in whom the diagnosis of syphilis was doubtful. In each case the test has been performed with an antigen of simple alcoholic extract at 37°C., a cholesterinized alcoholic extract at 37°C. and a simple alcoholic extract at 8°C. (ice-box temperature).

The tubes for the tests at 37°C. were set up in the same rack, four tubes being used for each serum: Tube 1 received 0.04 cc. of the patient's serum, 0.2 cc. of complement, 0.1 cc. of cholesterinized antigen and 0.3 cc. of salt solution. Tube 2 received 0.02 cc. of the patient's serum, 0.2 cc. of complement, 0.1 cc. of cholesterinized antigen and 0.3 cc. of salt solution. Tube 3 received 0.04 cc. of the patient's serum, 0.1 cc. of simple antigen and 0.3 cc. of salt solution. Tube 4 received 0.08 cc. of the

patient's serum, 0.2 cc. of complement and 0.3 cc. of salt solution. This information is presented in schematic form in table 1. After thorough shaking and incubation at 37°C. for one hour, 0.4 cc. of the suspension of sensitized sheep cells was added to each tube and the racks returned to the incubator. Readings were taken when the serum and antigen controls showed complete hemolysis, which was usually in from fifteen to thirty minutes.

TABLE 1  
*The test at 37°C.*

	TUBES			
	1	2	3	4
Patient's serum.....	0.04	0.02	0.04	0.08
Cholesterinized antigen.....	0.10	0.10		
Simple antigen.....			0.10	
Complement.....	0.20	0.20	0.20	0.20
Salt solution.....	0.30	0.30	0.30	0.30

For the test in the refrigerator, three, and more recently four tubes have been used for each serum to be tested. Tube 1 received 0.04 cc. of the patient's serum, 0.2 cc. of complement, 0.1 cc. of simple alcoholic antigen and 0.3 cc. of salt solution. Tube 2 received 0.02 cc. of the serum, 0.2 cc. of complement, 0.1 cc. of simple antigen and 0.3 cc. of serum. Tube 3, which has been only recently included in this test, received 0.04 cc. of serum, 0.2 cc. of complement, 0.1 cc. of cholesterinized antigen and 0.3 cc. of salt solution. Tube 4, the anticomplementary control, received 0.08 cc. of the patient's serum, 0.2 cc. of complement and 0.3 cc. of salt solution. After setting up the test, the racks were promptly transferred to the refrigerator, where they were left at least four hours and usually over night (16 to 18 hours) at 7° to 8°C. Table 2 is a diagrammatic representation of the ice-box test. Suspension of sensitized sheep blood cells, 0.4 cc., was then added to each tube and the lower part of the tubes immersed in a water bath at 40°C. The controls upon the sera and upon the antigens usually showed complete hemolysis in from ten to fifteen minutes. The readings were then made.

The sera of the patients were always inactivated at 56°C. just before use. The complement was always a 10 per cent guinea-pig serum in salt solution, freshly prepared and kept cold. The sensitized sheep blood cells were prepared by mixing a 1:20 suspension of fresh washed cells in salt solution with an equal volume of a solution of inactivated hemolytic rabbit serum of such strength that a mixture of 0.1 cc. of the complement to be used, 0.2 cc. of sheep cells, 0.2 cc. of the diluted amboceptor and 0.5 cc. of salt solution showed complete hemolysis at the end of fifteen minutes at 37°C. This hemolytic system was always titrated and the proper dilution of amboceptor ascertained just before each test. It will be noted that two units

TABLE 2  
*The test in the ice-box*

	TUBES			
	1	2	3	4
Patient's serum.....	0.04	0.02	0.04	0.08
Simple antigen.....	0.10	0.10		
Cholesterinized antigen.....			0.10	
Complement.....	0.20	0.20	0.20	0.20
Salt solution.....	0.30	0.30	0.30	0.30

of complement, 0.2 cc., were always employed in the tests. The antigens used were simple solutions in absolute ethyl alcohol of the alcoholic extractives of human heart, beef heart and guinea-pig heart (10 grams of heart muscle in 100 cc. of absolute ethyl alcohol) and these same solutions reinforced by the addition of 0.4 per cent of pure cholesterin. Just before use each antigen was diluted according to previous titrations with from 10 to 30 volumes of salt solution to produce a fine emulsion, of which 0.1 cc. would be the proper dose for each tube. We are not prepared, at present, to report upon the value of isolated lipoids or acetone insoluble fractions of the extractives.

In the period from April 8, 1916, to July 22, 1916, there have been tested by these methods, blood serum, spinal fluid or cerebral fluid of 477 patients. According to the available clinical



data, these have been divided into three main groups, known syphilitics, doubtful cases and non-syphilitic patients. The second group has been subdivided into those probably syphilitic and those probably not syphilitic.

Group I was made up of known syphilitics. Most of them gave definite histories of syphilis and the majority were under treatment for syphilis at the time of the test. Only one, No. 8694, was in the primary stage. Many were examples of latent syphilis and a considerable number had involvement of the central nervous system. There were 110 patients in this group, of whom 99 gave positive histories and 11 denied knowledge of infection, but presented unmistakable evidence of lues. Fifty

TABLE 3

*Summary of 127 Wassermann tests by three different methods upon known syphilitics*

TEMPERATURE OF FIRST INCUBATION	ANTIGEN	POSITIVE*		DOUBTFUL*		NEGATIVE*	
		Number	Per cent	Number	Per cent	Number	Per cent
<i>deg. C.</i>							
37	Cholesterinized	74	58.2	10	7.9	43	33.8
37	Simple	41	32.2	1	0.8	85	66.9
8	Simple	98	77.1	5	4.1	24	18.8

\* Interpretation of terms:

Positive: 4+, complete fixation; 3+, about 75 per cent fixation;  
2+, about 50 per cent fixation.

Doubtful: 1+, about 25 per cent fixation; =, less than 25 per cent fixation.

Negative: —, no fixation.

of these patients were free from manifestations of syphilis at the time of the tests. The results of the 127 tests on these 110 patients are summarized in table 3.

It is at once evident that in this group of known syphilitics, the simple antigen used at 37°C. gave a negative result in about two-thirds of the cases, the cholesterinized antigen at 37°C. gave a negative result in about one-third, while the simple antigen at 8°C. gave a negative result in only 18.8 per cent, less than one-fifth. So far as this series of 127 tests goes, the third method would appear much the most satisfactory. The detailed data in regard to these 127 specimens are briefly presented in table 4.

TABLE 4

*Brief data concerning 127 specimens of blood serum, spinal fluid and cerebral fluid from 110 cases of syphilis. Group I*

LABORATORY NUMBER	INFECTION	TREATMENT	MANIFESTATIONS OF DISEASE AT TIME OF TESTS	COMPLEMENT FIXATION		
				At 37°C.		At 8°C.
				Cholester- inized.	Simple	Simple
7571			None	—	—	2
7576			None	—	—	1
7601	Hereditary	None	Interstitial keratitis	4	±	4
7602	12 years ago		Headache; pains in legs	—	—	3
7607			None	—	—	—
7631	Hereditary	None	Imbecility	4	—	±
7632	3 years ago	None	Osteitis, tibia	4	4	4
7634	3 years ago		None	—	—	—
7636			None	4	—	4
7640			None	4	—	4
7659		None	Hemiplegia	4	—	4
7670	9 years ago	None	Vertigo	—	—	±
7673	12 years ago	Hg and KI	Nausea	—	—	4
7674	November, 1915	Hg and 606	None	—	—	4
7678			Tabes	—	—	2
7687			Paresis	—	—	4
7689†			Paresis	4	—	4
7690	3 years ago		None	—	—	—
7691	3 years ago		None	—	—	4
7694			Sore throat	4	—	4
7696	5 months ago		None	4	4	4
7698		606	None	—	—	—
7700	1 year ago		None	4	—	4
7701	4 years ago		None	—	—	±
7702	13 years ago		None	—	—	—
7703	18 months ago		None	—	—	—
7730	15 years ago		None	1	—	3
7731	Husband is luetic		Syphiloderm	4	—	4
7732		None	Gumma, leg	2	—	4
7738		Hg	Paresis	4	4	4
7739		606 and 914	Paresis	—	—	—
7741†		Hg	Paresis	4	—	2
7743	Hereditary	None	Juvenile paresis	4	—	4
7747			Ulcer, throat; deaf- ness	±	—	3
7750			None	—	—	—

TABLE 4—*Continued*

LABORATORY NUMBER	INFECTION	TREATMENT	MANIFESTATIONS OF DISEASE AT TIME OF TESTS	COMPLEMENT FIXATION		
				At 37°C.		At 8°C.
				Cholester- inized	Simple	Simple
7761	8 months ago	Until 2 months ago	None	3	—	2
7762	10 years ago	Until 2 months ago	None	—	—	4
7763	2 years ago	Until 6 weeks ago	None	—	—	—
7778	2 years ago		Encephalitis	4	2	2
7797			None	4	—	4
7807			Arterio-sclerosis and myocarditis	4	4	4
7808			None	±	—	4
7810			None	4	—	4
7821*			Arterio-sclerosis and myocarditis	4	4	4
7829			Encephalitis	±	—	4
7830†	1 year ago		Encephalitis	±	—	4
7835			None	4	—	4
7839			None	4	4	4
7846			Encephalitis	4	4	4
7847†			Encephalitis	4	4	4
7850	12 years ago	Until 2 years ago	None	—	—	3
7853	1914	Hg	None	—	—	2
7857	10 years ago	For 2 years	Arthritis	—	—	2
7859	1 year ago	Arsenobenzol	None	—	—	—
7866	1 month ago		None	1	—	2
7867	6 weeks ago		None	4	4	2
7874			None	4	—	4
7922			Paresis	4	4	4
7933			Aortic regurgita- tion	4	4	4
7935*			Paresis	—	—	—
7970			Arthritis	4	4	4
7974			Encephalitis	4	±	4
7992			Tabes	4	4	4
8008			None	1	—	—
8009	Congenital (6 years old; brother of 8010)	None	Retardation	4	4	4

TABLE 4—Continued.

LABORATORY NUMBER	INFECTION	TREATMENT	MANIFESTATIONS OF DISEASE AT TIME OF TESTS	COMPLEMENT FIXATION		
				At 37°C.		At 8°C.
				Chol- ester- inized	Simple	Simple
8010	Congenital (5 years old; sister of 8009)	None	Retardation	—	—	—
8012			None	4	4	4
8017	6 years ago		None	4	—	—
8026	Husband (7992) has tabes		None	4	—	±
8050			Tabes	4	—	4
7785			Cerebro-spinal syph- ilis	4	4	4
8052			Paresis	4	—	4
8055			Tabes	—	—	—
8056†			Paresis	4	4	4
8061	11 months ago		None	—	—	—
8066			Cerebro-spinal	4	4	4
8071			Cerebro-spinal	4	2	4
8072‡			Paresis	—	—	4
8074			Tabes	4	—	4
8085			Sore throat	4	4	4
8090	1 year ago		None	—	—	4
8092			Frequency of uri- nation	—	—	4
8096	None	None	Gumma of tongue	4	4	4
8101	6 months ago		Pains in chest	4	—	4
8113	15 years ago		Osteomyelitis	±	—	—
8114	8 years ago		Folliculitis	±	—	—
8117	3 years ago		None	—	—	—
8121			None	4	4	4
8223			None	4	4	4
8226			None	4	4	4
8228		None	Paresis	4	4	4
8255	10 years ago		None	4	4	4
8260	20 years ago		None	—	—	—
8267†		None	Paresis	4	4	4
8272		None	Rash; adenopathy	4	—	4
8276	1914	Hg	None	2	—	2
8277	1905	Hg	None	—	—	—
8288*			Tabes	—	—	—
8290	5 years ago		Phlebitis	4	4	4

TABLE 4— *Concluded*

LABORATORY NUMBER	INFECTION	TREATMENT	MANIFESTATIONS OF DISEASE AT TIME OF TESTS	COMPLEMENT FIXATION		
				At 37°C.		At 8°C.
				Chol- ester- inized	Simple	Simple
8293*			Paresis	4	4	4
8295			Paresis	—	—	4
8296			Tabes	—	—	4
8297			Paresis	—	—	4
8405		None	Large sore in mouth	4	4	4
8411	10 years ago		Headaches	—	—	2
8412	14 years ago	None	None	—	—	1
8420		None	None	4	—	4
8421			Orchitis	4	4	4
8446			Rash	4	4	4
8449			Headaches; pains in legs	—	—	4
8450			Paresis	3	—	4
8694	3 weeks ago	None	Sore on penis	4	2	3
8696	24 years ago	Hg and KI	Ulcers, nose	4	—	4
8697		606	None	1	—	—
8701	8 years ago	None	Aortic regurgita- tion	4	—	3
8704	Congenital	None	Gumma, right fore- head	4	4	4
8705			Paresis	—	—	4
8706			Paresis	2	—	3
8742	25 years ago	Hg	Gumma of turbi- nate	2	4	4
8745	None	None	Ulcer of turbinate	4	4	4
8746	5 months ago	606	Optic neuritis	4	2	4
8747	1915	Hg	Adenitis	3	2	4
8752	None	None	Cerebro-spinal lues	2	—	4
8763†	None	None	Cerebro-spinal lues	2	2	4
8764†			Cerebro-spinal	—	—	—
8765			Cerebro-spinal	3	—	4

\* A simple number indicates blood serum. A number with asterisk indicates a duplicate specimen of blood serum from a patient previously tested. Specimen 7821\* is from the same patient as 7807; 7935\* as 7739; 8233\* as 7992; 8293\* as 8066; 8295\* as 7847.

† A number followed by this character designates a spinal fluid. The patient's blood serum was usually tested also. The following numbers indicate spinal fluid and blood serum, respectively, from the same patient: 7639† and 7687; 7741† and 7738; 7830† and 7829; 7847† and 7778; 8056† and 8052; 8267† and 8228; 8763† and 8752; 8764† and 8765.

‡ A number followed by this character designates a fluid from the cerebral ventricles. The patient's blood serum was usually tested also. Cerebral fluid 8072‡ was from the same patient as blood serum 8066; 8297‡ from the same as 8293.

Group II is in part made up of those patients for whom a clinical diagnosis of syphilis could not be made, but whose history, physical condition or response to treatment indicated a probable diagnosis of syphilis. They presented such conditions as interstitial keratitis, unilateral painless swelling of the tonsil, perforation of the hard palate, paralysis of the external ocular muscles, transverse myelitis, aortitis, periostitis, arthritis, epididymitis, retarded mental development and so-called nervousness. After consideration of the history, physical examination and response to treatment, this group has been subdivided into Group II A, probably syphilitic, and Group II B, probably not syphilitic. All of these cases could not be followed, but if, after a negative Wassermann, the patient has improved on anti-

TABLE 5  
*Summary of 44 Wassermann tests by three different methods upon patients probably syphilitic*

TEMPERATURE OF FIRST INCUBATION	ANTIOEN	POSITIVE		DOUBTFUL		NEGATIVE	
		Number	Per cent	Number	Per cent	Number	Per cent
<i>deg. C.</i>							
37	Cholesterinized	29	65.9	2	4.6	13	29.5
37	Simple	16	36.3	1	2.4	27	61.3
8	Simple	33	75.0	0	0.0	11	25.0

syphilitic treatment he has been classed in Group II A. On the other hand, if, even after a positive Wassermann, the patient has not improved on antisyphilitic treatment, but has responded to other treatment, as, for example, a case of arthritis to salicylates, he has been classed in Group II B, probably not syphilitic. Of course, this division can be only approximately accurate. There is always the possibility of coincident syphilis quite independent of the presenting signs and symptoms.

The results of the 44 tests on the 43 patients in Group II A are summarized in table 5. Again, the results obtained with the cholesterinized antigen at 37°C. are intermediate and the extremes are occupied by the simple antigen at the two different temperatures. Assuming that nearly all these patients were syphilitic, the test at 8°C. would again appear to be the most satisfactory. The detailed data in regard to these 44 specimens are briefly presented in table 6.

TABLE 6

*Brief data concerning 44 specimens of serum of 43 cases probably of syphilis.  
Group II A*

LABORATORY NUMBER	MANIFESTATIONS OF DISEASE AT TIME OF TESTS	DIAONOSIS	COMPLEMENT FIXATION		
			At 37°C.		At 8°C.
			Cholester- inized	Simple	Simple
7569	Nervousness; headache		—	—	2
7603	Swollen tonsil	Lues	4	4	4
7606	None (has given + Wass.)	Latent lues	—	—	—
7609	Paralysis of external recti		4	—	3
7639	Tinnitus aurium		—	—	3
7686	Transverse myelitis	Congenital lues	—	—	—
7742	Chronic inflammation of tongue		4	2	4
7796	Interstitial keratitis		4	—	4
7800	None (under treatment)	Lues	—	—	—
7819			4	4	4
7826	Vomiting	Pregnancy	4	—	4
7836	None (infection (?) 15 years ago)	Lues (treated)	—	—	—
7838	None (infection (?) 1½ years ago)	Lues (treated)	—	—	—
7842	Cholecystitis		3	—	4
7851*	Transverse myelitis	Congenital lues	1	—	—
7856	Stiff knee		—	—	4
7920	Paralytic strabismus		4	—	4
7934	Arteriosclerosis		4	4	4
7959	Headache		4	4	2
7960	Ulcer of foot		—	—	4
7962		Neurasthenia	4	4	4
7965	Headache; weakness		4	4	4
7966	Arthralgia	Lues	4	4	2
7967	Sleeplessness; nervousness		4	4	2
7968	Rash on body		4	2	—
7972	Spastic weakness, left leg		4	4	4
7973	None		4	4	4
8022		Diabetes	4	—	4
8031	Ulcer of nasal septum	Lues	4	—	4
8038	None		4	—	4
8047		Duodenal ulcer	4	—	4
8062	Swollen testis		—	—	—
8069	Abdominal pains		±	—	2
8105		Double aortic lesion; 4 mis-carriages; 1 insane child	—	—	4

TABLE 6—*Continued*

LABORATORY NUMBER	MANIFESTATIONS OF DISEASE AT TIME OF TESTS	DIAGNOSIS	COMPLEMENT FIXATION		
			At 37°C.		At 8°C.
			Cholester- inized	Simple	Simple
8116		Endocarditis	4	2	4
8135	Abdominal colic and bloody urine, 3 attacks over 12 years		4	±	4
8252	Osteomyelitis		4	2	4
8258	Sore nose and throat		4	—	—
8265	Pain in shoulder; ataxia		4	—	—
8266	History positive, 15 years ago, treated	Duodenal ulcer	—	—	—
8280	Sore nose and throat		—	—	2
8442	Enlargement of thyroid	Simple goitre	4	3	4
8447		Endocarditis and myocarditis	4	—	4
8748	Cervical adenitis		3	4	4

\* Specimen 7851\* was from the same patient as 7686, both being blood sera.

The results of the 60 tests on the 59 patients in Group II B are presented in summary in table 7. The disparity between the percentage of positive reactions given by the cholesterin-reinforced antigen and by the simple antigen is very striking. Note

TABLE 7

*Summary of 60 Wassermann tests by three different methods upon patients probably not syphilitic*

TEMPERATURE OF FIRST INCUBATION	ANTIGEN	POSITIVE		DOUBTFUL		NEGATIVE	
		Number	Per cent	Number	Per cent	Number	Per cent
<i>deg. C.</i>							
37	Cholesterinized	24	40.0	9	15.0	27	45.0
37	Simple	1	1.6	0	0.0	59	98.3
8	Simple	3	5.0	3	5.0	54	90.0

also that the one positive reaction given by the simple antigen at 37°C. and the three given at 8°C. were only 2 + positives, while, on the other hand, 18 of the 24 positives given by the cholesterin-reinforced antigen were examples of complete fixation.



Let it be recalled that these cases are classed as probably not syphilitic. It is quite possible that some of them are really luetic, but there is no definite evidence of lues in the histories or in the physical findings. Case 7963—that of an imbecile child—is highly suggestive. The serum gave total fixation with the cholesterin-reinforced antigen at 37°C., but no fixation with the simple antigen, either at 37°C. or at 8°C. We were so fortunate as to obtain the sera of both parents and neither gave any degree of fixation by either method, nor did they give any history of syphilis.

It is quite possible that if we had been able to follow up cases 8003, 8004, 8005, 8013 and 8015 in a similar manner, we might have obtained results very like those found in case 7963. If patients in this group are considered to be luetic they are so considered upon the evidence of the Wassermann reaction alone and, moreover, upon a Wassermann reaction performed with a cholesterin-reinforced antigen only. In view of the fact, which has been demonstrated in the consideration of Groups I and II A, that the simple antigen at 8°C. is far more sensitive in known syphilitics and in probable syphilitics than is the cholesterin-reinforced antigen at 37°C., we prefer to consider these cases as probably not syphilitic and we regard the positive reactions obtained in this group as false positives. It is very apparent that the cholesterin-reinforced antigen at 37°C. is far more culpable than the simple antigen at either temperature. Brief data in regard to cases in Group II B are presented in table 8.

Group III is made up of those cases which we consider to be certainly not syphilitic. We feel that lues has been satisfactorily ruled out in most, if not all, of the cases classed in this group.

The results of the tests on the patients in Group III are summarized in table 9. The greater number of these were patients in the medical wards, where the Wassermann reaction is made a routine procedure. Negative reactions were obtained in all these cases by all three methods. It is unnecessary to append detailed data on this long list of cases. Suffice it to say that none of them gave the least evidence of lues either in history, symptoms or signs.

TABLE 8  
Data concerning 60 specimens of serum of 59 cases, probably not of syphilis.  
Group II B

LABORATORY NUMBER	CLINICAL DATA	DIAGNOSIS	COMPLEMENT FIXATION		
			At 37°C.		At 8°C.
			Cholesterolized	Simple	Simple
7570	Periostitis of metacarpals	Tuberculosis	4	—	—
7600	No manifestations of disease; father luetic?	Lues (?)	—	—	—
7612	Nervousness	Neurasthenia	—	—	±
7628	Swelling in scrotum	Epididymitis	4	—	—
7638	Delayed healing, in boy of 9 years		4	—	—
7648	Follicular tonsillitis and acute arthritis	Streptococcus disease	4	—	—
7660	Intense jaundice; splenectomy done	Hemolytic jaundice	4	—	—
7661	Chronic endocarditis		4	—	±
7675	Chronic diarrhea	Lues (?)	—	—	—
7692	Questionable history of infection 35 years ago; no treatment	Syphilophobia	—	—	—
7695	Headache; miscarriage; no treatment	Lues (?)	—	—	—
7697	2 miscarriages; husband luetic (?)	Lues (?)	—	—	—
7699	Lesion of skin of palm	Lues (?)	—	—	—
7715	Aortitis; enlarged liver; jaundice; 3 miscarriages. No treatment	Lues	—	—	2
7764	Mental deficiency		±	—	—
7765	No manifestations; history questionable		—	—	—
7787*	(Case 7715; second specimen)		±	—	—
7794	No manifestations; history questionable		—	—	—
7834	Sore on penis of 6 weeks duration		—	—	—
7848	Unioocular blindness		—	—	—
7849	Mental deficiency; father luetic (?)		—	—	—
7860	Miscarriages		—	—	—
7862	Periostitis of tibia; arthritis of knee		—	—	—
7863	Headache; questionable history of lues		—	—	—

\* Specimen 7787\* was blood serum from the same patient as specimen 7715.

TABLE 8—Continued

LABORATORY NUMBER	CLINICAL DATA	DIAGNOSIS	COMPLEMENT FIXATION		
			At 37° C.		At 8° C.
			Cholesterol- inized	Simple	Simple
7917	Mental deficiency	Paresis (?)	2	—	—
7921			—	—	—
7963	Imbecile		4	—	—
8206	Mother of 7963		—	—	—
8207	Father of 7963		—	—	—
7988	Myocardial deficiency		—	—	±
7964	Pain in head		±	—	—
7969	Paralysis of right vocal cord		±	—	—
7971	Pain in knees	Syringomyelia	±	—	—
7982			4	—	—
7983		Carcinoma of stomach	4	—	—
7984		Carcinoma of stomach	±	—	—
7986	Pleurisy with effusion	Streptococcic bacteremia	±	—	—
7989	Endocarditis		2	2	2
8000	Mental retardation		4	—	—
8003	Mental retardation		4	—	—
8004	Mental retardation	Tuberculide	±	—	—
8005	Mental retardation		4	—	—
8007	Skin lesion		4	—	—
8013	Mental retardation		2	—	—
8015	Backward		4	—	—
8030	Epilepsy		4	—	—
8032	Questionable history of infection 2 years ago		—	—	—
8035	Skin lesion	Lichen planus	—	—	—
8049	Endocarditis, malignant; (autopsy)		4	—	—
8119	Retinitis; questionable history of lues		—	—	—
8120	Epilepsy	Gonococcal infection	—	—	2
8227	Acute tonsillitis; acute nephritis		±	—	—
8246	Lupus		4	—	—
8250	Keratitis; history questionable		—	—	—
8251	Questionable history of lues		—	—	—
8254	Idiocy		2	—	—
8264	Epigastric pain and vomiting		2	—	—
8268	Tinnitus, staggering, vomiting		—	—	—
8300	Pain and swelling in knee		4	—	—
8453	Mental retardation		2	—	—

TABLE 9

*Summary of 265 Wassermann tests by three different methods upon patients certainly not syphilitic*

TEMPERATURE OF FIRST INCUBATION	ANTIGEN	POSITIVE		DOUBTFUL		NEGATIVE	
		Number	Per cent	Number	Per cent	Number	Per cent
<i>deg C.</i>							
37	Cholesterinized	0	0	0	0	265	100
37	Simple	0	0	0	0	265	100
8	Simple	0	0	0	0	265	100

## SUMMARY

1. In a group of 110 syphilitic individuals in various stages of the disease, the cholesterin-reinforced antigen with incubation at 37°C. gave 58.2 per cent, the simple antigen at 37°C., 32.2 per cent, and the simple antigen at 8°C., 77.1 per cent of positive tests.

2. In a group of 43 patients, probably syphilitic, the first method yielded 65.9 per cent, the second 36.3 per cent and the third method 75.0 per cent of positive fixations.

3. In a group of 59 patients, probably not syphilitic, the first method yielded 40.0 per cent, the second 1.6 per cent and the third 5.0 per cent of positive tests.

4. In a group of 265 non-syphilitic patients, tests by all methods were negative throughout.

## CONCLUSIONS

The use of a simple alcoholic antigen, with the first incubation carried out in the ice-box for four to twenty-four hours, is more sensitive in the detection of syphilis than the other procedures tested. Furthermore, a positive result thus obtained is much more trustworthy evidence of syphilis than is a positive fixation with a cholesterinized antigen.

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## STUDIES IN ANAPHYLAXIS

### XVIII. THE MECHANISM OF DELAYED SHOCK

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It may now be regarded as a well established fact that cellular antibody alone is responsible for the reaction known as acute anaphylactic shock. There are, however, certain other manifestations of anaphylaxis which are with difficulty explained upon that basis, and on this account many conservative workers incline to the belief that under certain circumstances circulating antibody may also play an important part in the production of the symptoms. At first sight it might not appear to be a matter of great consequence whether the interaction between antigen and antibody takes place within the cells, or in the fluids of the body. Such, however, is far from the fact. The humoral theory requires the assumption of an intermediary toxic product, a hypothetical anaphylatoxin, which secondarily affects the cells. The cellular theory does not require such an hypothesis. Moreover, the humoral theory is committed to the belief that circulating antibody may exercise either a protective or an injurious function, depending upon various accidental circumstances; the cellular theory is strictly correlated with the view that circulating antibody is invariably protective, and cellular antibody invariably the agent of anaphylactic symptoms. Thus it appears that the significance of anaphylatoxins, and also the mechanism of immunity, will eventually be largely determined according to the solution of the problem here under discussion. Doubtless the extensive literature on the mode of production of the anaphylactic response has been stimulated by a tacit recognition of these implications. There are two principal

arguments in favor of the participation of circulating antibody in anaphylaxis, namely the phenomenon of delayed shock, and the production of anaphylactoid symptoms by the simultaneous injection of antigen and antibody. In the present paper the former of these arguments is studied experimentally.

#### ON THE DELAYED REACTION IN ANAPHYLAXIS

The term "delayed reaction" is used to indicate the fact that a considerable interval of time elapses between the injection of antigen into a sensitized animal and the occurrence of the symptoms induced thereby. Usually this interval is brief, covering from five to thirty minutes; the symptoms are, as a rule, milder than those which follow intravenous injections, although death not infrequently supervenes. The delayed reaction occurs most typically when the intoxicating injection is given intraperitoneally. After intravenous injections it occurs only exceptionally, and then under certain special circumstances which will be subsequently described.

An excellent description of delayed shock is given by Anderson (1) in the following words.

Within five or ten minutes, when the injection is given intraperitoneally, the guinea pig becomes restless and agitated; it runs about the cage and sometimes utters sounds of distress; then there appear manifestations of peripheral irritation and respiratory embarrassment, as shown by scratching at the mouth, coughing, sneezing, rapid and irregular respiration. An exceedingly characteristic feature of the respiratory involvement is that at intervals the animal makes an unusually deep inspiratory effort with the diaphragm, resulting in a marked sinking at the lower end of the sternum. The stage of excitement is soon followed by one of paresis, or in some cases complete paralysis. The animal is unable to stand and if it attempts to do so it falls upon its side.

Guinea pigs in a condition of complete paralysis may fully recover, but within a short time convulsions usually begin and are almost invariably the forerunner of a fatal termination. Occasionally in guinea pigs not very susceptible the onset of the symptoms following an intraperitoneal injection may be delayed thirty or forty minutes, but in



only a few instances have I noted the onset of symptoms delayed as long as an hour.

In dogs, according to Richet (2) anaphylactic shock is regularly delayed.

This group of symptoms contrasts strikingly with that induced by the intravenous injection of a lethal dose of antigen in a well sensitized guinea pig. In the latter experiment the animal goes into immediate convulsions, and death usually occurs within five minutes. This result, however, does not always follow even after intravenous injections. If the guinea pig is not adequately sensitized the symptoms are very likely to be delayed and modified, and may then correspond closely with those above described by Anderson.

The significance of the delayed reaction depends upon the fact that the difference in onset and in manifestations as compared with acute shock has been attributed to a difference in mechanism. Acute shock has finally been demonstrated to result entirely from the reaction of fixed, or cellular, antibody to the introduced antigen. This fact Doerr (3) in his last review of anaphylaxis, freely grants; but he intimates, at the same time, that possibly the delayed response<sup>1</sup> may in whole, or in part, be mediated by a reaction between the free, or circulating, antibodies and the introduced antigen, with the production of toxic products, the so-called anaphylatoxins. This view, to which others have subscribed, postulates essentially different mechanisms for the immediate and the delayed forms of shock. Moreover, it has been claimed that acute anaphylactic shock, following upon the sudden flooding of the organism with an overwhelming dose of antigen, is the result of peculiar and artificial conditions such as exist only in experiment. The delayed type of shock, however, is asserted to represent the mode of reaction which probably obtains under the usual conditions of

<sup>1</sup> "It is possible that the toxic effects of protein digestion products may play a rôle in that form of the anaphylactic reaction in guinea pigs or in rabbits which does not lead to death within a few minutes, but which appears after a somewhat prolonged period of incubation, ending either in recovery or in death after a few hours."—Doerr.

infectious disease. From this reasoning it would follow that the symptoms of disease result primarily from the interaction between circulating antibody and antigen, to the practical exclusion of the cellular mechanism. For these reasons, herefore, it is of importance to determine the mechanism of delayed shock with accuracy.

Up to the present time, despite a considerable amount of speculative consideration of the subject, no attempt has been made to approach this problem by direct experiment. The present study details a series of experiments which were devised in the hope of throwing light on the mechanism of the delayed reaction.

The problem has been approached by two methods of study. In the first place, the attempt has been made to induce shock in the living animal under such conditions as would secure the exclusion either of circulating or of cellular antibody. In the second place, the Dale method has been modified in such wise as to permit the study of the isolated uterus under conditions similar to those which produce delayed shock in the living animal.

#### EXPERIMENTS ON SENSITIZED GUINEA PIGS

Delayed shock is typically induced in the sensitized animal by the intraperitoneal administration of antigen. Under such conditions it is not possible to determine what part of the result is contributed by the fixed antibodies, and what part by the circulating antibodies. It is, however, possible to conceive of a crucial experiment in this problem of the mechanism of the delayed reaction. If a guinea pig could be so prepared that all of the sensitizing antibodies were fixed in the cells, while none were in circulation, the occurrence of the delayed reaction would necessarily indicate a cellular mechanism for that type of response. If a guinea pig could be prepared in the reverse fashion, all of the antibodies being in circulation and none being fixed in the cells, the occurrence of the delayed reaction would, of course, indicate a humoral mechanism. Conditions are such that an absolutely ideal segregation of antibodies in the organ-

ism, in the manner above suggested, is not practicable. Nevertheless it can be experimentally approximated, to such an extent that the results have practically the validity of the ideal experiment.

*Exclusion of circulating antibody. Transfusion*

The guinea pigs employed in the following experiment had been actively sensitized by an injection of horse serum given at periods varying from six to seventeen months previously. Presumably, at the time chosen for the injection of the antigen, all antibody had disappeared from the circulation. In order to further insure this result, these animals were all bled from the carotid, as much blood being taken as could be obtained by this procedure. The bleedings ranged in amount from 5 cc. to 8.5 cc. An equal quantity of normal guinea pig blood in 1 per cent sodium citrate, according to a method previously described (4), was injected into the external jugular to replace the blood so drawn. In order to ascertain whether antibodies were present in the blood taken from the sensitized animals, the total serum of each bleeding was injected intraperitoneally into a normal guinea pig, and the latter was tested after an interval of two days by the injection of the original antigen, horse serum. None of this latter group of animals, however, responded in the least degree to the injection of the horse serum. It is evident, therefore, that the group of guinea pigs that had been sensitized some months previously did not retain any demonstrable antibody in their circulation at the time of the experiment. The replacement of their own blood in considerable part by normal guinea pig blood offers an additional guarantee that their blood was practically free from antibody. These guinea pigs, then, were utilized for the intraperitoneal injection of antigen. In accordance with the experience of Rosenau and Anderson, who found that actively sensitized animals remain hypersusceptible for the remainder of their lives, a larger proportion of these animals responded anaphylactically to these injections. The data of the experiment are given in the following table, which does not, however, include those guinea pigs which failed to react.

TABLE 1

GUINEA PIG	SENSITIZATION BY HORSE SERUM	ANTIBODIES IN BLOOD	BLOOD WITH- DRAWN	HORSE SERUM I. P.	RESULT
1	0.1 cc. 6 months previously	Negative	cc. 6	cc. 5	Delayed reaction. Recovery in 4 hours
2	0.2 cc. 8 months	Negative	5	5	Death in 1 hour
3	0.1 cc. 12½ months	Negative	7	5	Delayed reaction. Found dead in morning
4	0.05 cc. 14 months	Negative	6.5	5	No marked reaction. Cough- ing and sneezing
5	0.1 cc. 17 months	Negative	8.5	5	Delayed reaction. Death in 1½ hours

In these animals, therefore, the delayed reaction supervened, in spite of the absence of any demonstrable antibodies in the blood. It seems necessary to conclude that the reaction was due to the activity of the fixed, or cellular antibodies.

*Exclusion of the circulating antibody. Utilization of the latent interval of passive sensitization*

If a guinea pig is given an intravenous injection of antibody, such an animal does not become passively sensitized for hours. It may respond slightly to the intravenous administration of antigen after about two hours; but it will not manifest any reaction to an intraperitoneal injection for about twelve hours or more. It has been demonstrated that this so called latent period covers the time required for the fixation of the introduced antibody by the cells. If the intraperitoneal injection of antigen is given very shortly after the intravenous injection of antibody, therefore, it is quite certain that the conditions are such as to exclude the possible participation of the body cells in any reaction which may occur. On the other hand, if the production of a delayed reaction, as usually seen after the intraperitoneal in-

jection of antigen into a sensitized animal, is mediated by the circulating antibody, exactly the same result ought logically to follow in this experiment, the conditions being appropriate therefor.

In performing the experiment certain preliminary conditions must be fulfilled. Most important of these is the possession of an antiserum which may be given intravenously in large doses. The following experiments have all been done with the serum of a rabbit immunized against horse serum. Certain rabbits yield an antiserum of this type which is toxic to guinea pigs when given in amounts of even less than 1 cc. intravenously. What conditions produce this toxicity is not known. During the course of this work several animals had to be discarded because of this disturbing element. Other rabbits yielded an antiserum of even higher titer, which was well borne in such quantities. Having secured a satisfactory antiserum, it is necessary to determine first what amount will passively sensitize a guinea pig in such a manner that the intraperitoneal injection of antigen produces characteristic anaphylactic symptoms, though not necessarily death. This amount ordinarily is several times greater than the minimal amount necessary to prepare guinea pigs for anaphylactic death through the intravenous administration of antigen. The following protocols illustrate a preliminary set of experiments.

TABLE 2

GUINEA PIG	SENSITIZING INJECTION INTRA- VENOUSLY	TOXIC INJECTION NEXT DAY INTRAPERI- TONEALLY	RESULTS
	cc.	cc.	
1	0.3	2.5	No symptoms
2	0.5	2.5	After 20 minutes paretic, atactic. Mild convulsions. Recovered in 2 hours
3	0.5	2.5	Similar to above
4	0.7	2.5	Respiratory and cutaneous symptoms. Severe prostration. Died during day
5	0.8	2.5	Died in 40 minutes
6	1.0	2.5	Immediate dyspnea and paresis. Convulsions; death in 12 minutes
7 8	} 2.0	2.5	Very mild symptoms

In this series the sensitizing serum was derived from Rabbit 894, immunized to horse serum; 0.15 cc. of this serum sensitized guinea pigs passively so that the intravenous injection of 0.05 cc. of horse serum caused immediate anaphylactic death.

It is evident that the preliminary injection of 0.5 cc. of the serum of Rabbit 894 passively sensitizes guinea pigs so that they present unmistakable mild and delayed symptoms upon the subsequent injection of 2.5 cc. of horse serum intraperitoneally. One cubic centimeter of the immune serum sensitizes much more effectively, and may be taken as the optimal sensitizing dose. (Larger injections of the immune serum, such as 2 cc., leave so considerable a residue of antibody in the circulating blood that the animal is protected against the anaphylactic effects of peritoneal injections of antigen. Weil (5)).

The second stage of the experiment consists in immediately following the intravenous injection of the immune rabbit serum by the intraperitoneal administration of the horse serum. Upon the assumption that delayed shock is due to the interaction of antigen and antibody in the blood, this experiment offers the proper conditions for its production. The following protocols illustrate the effect of this procedure.

TABLE 3

GUINEA PIG	SENSITIZING INJECTION INTRAVENOUSLY	ANTIGEN INJECTION SIMULTANEOUSLY INTRAPERITONEALLY	SYMPTOMS
	cc.	cc.	
1	0.4	2.5	None
2	0.5	2.5	None
3	0.7	2.5	None
4	0.7	2.5	None
5 }	1.0	2.5	None
6 }			

It seems quite clear from these experiments that the circulating antibody cannot be a factor in the production either of mild or of delayed shock. The only conclusion permitted by the facts is that the cellular antibodies are responsible for delayed shock exactly as they are for acute shock.

It would appear, therefore, that delayed shock is induced

under conditions which insure the somewhat gradual access of antigen to the body cells, a condition which is fulfilled when the antigen is given intraperitoneally. There is, however, one important modification of this statement. If the guinea pigs are given an amount of immune rabbit serum which is not sufficient to sensitize them thoroughly, the *intravenous* injection of antigen will give rise not to acute, but to moderate and delayed shock. Therefore, one may conclude that acute shock is induced only when sufficiently large amounts of cellular antibody and of introduced antigen are suddenly brought into contact. If either of these factors be insufficient in amount, or if the contact be very gradual instead of sudden, a mild and delayed type of shock may result.

#### EXPERIMENTS ON THE ISOLATED UTERUS

If it be true that cellular antibody is the agent in delayed shock, it ought to be possible to demonstrate delayed shock in the isolated uterus by means of properly devised methods. In view of the fact that delayed shock is produced in the living animal by the intraperitoneal injection of antigen, which insures gradual absorption, it seemed possible that the same result might be achieved in the Dale preparation by securing the slow and gradual addition of the antigen to the bath in which the uterus is suspended. In order to test this idea, the uterus of a guinea pig sensitized toward horse serum was suspended in the usual manner. Above the apparatus a burette was fastened, and so regulated as to deliver drop by drop at the edge of the container. This burette was filled with a 1 per cent solution of horse serum in normal salt. The diffusion of the horse serum through the fluid is naturally slow, so that it first reaches the uterine preparation only in extremely dilute condition. On the other hand, the total concentration of horse serum in the bath is constantly on the increase. The conditions present a not imperfect parallel to that which obtains in the blood following upon the intraperitoneal introduction of antigen. The figures illustrate the results obtained by this method of procedure.

Figure 1 shows a preliminary slight response after 1 cc. of 1 per cent horse serum had run in. This appeared five minutes after the flow had started, and gradually subsided within seven minutes. With 4.5 cc., seventeen minutes after starting the flow, a gradual rise began, which reached its maximum about one hour from the start.

In figure 2, the rise began about twenty minutes after the flow had started. There was a gradual ascent, followed by a sustained contraction lasting about three-quarters of an hour.

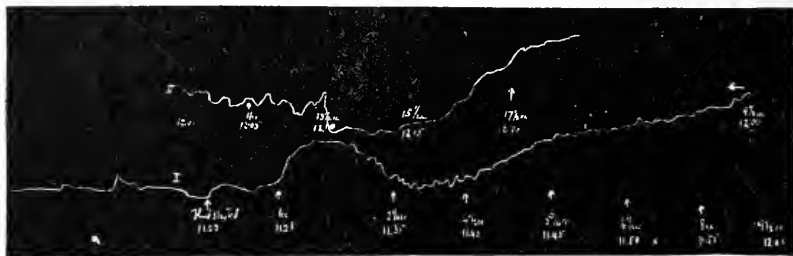


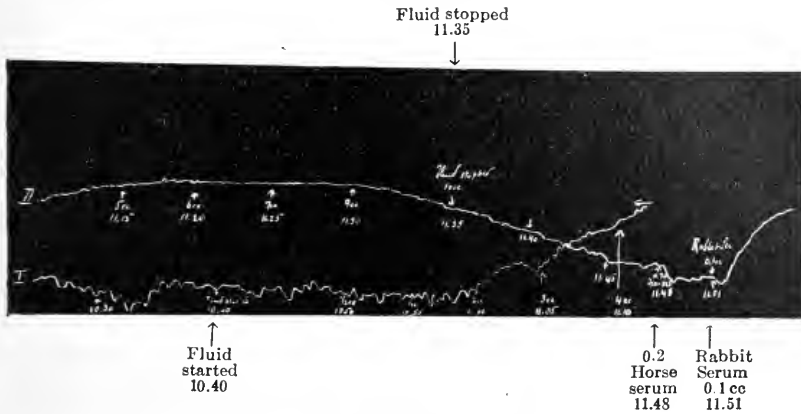
FIG. 1. GUINEA PIG 418. JANUARY 26

On December 23, this animal had been sensitized by an intraperitoneal injection of 1 cc. of horse serum. One per cent horse serum was run into the bath from the burette. The level of the drum was not changed during the experiment, so that Line II, which is to be read as the direct continuation of Line I, represents the increase in muscular tonus. Figure 5 is the tracing of the opposite horn of this organ.

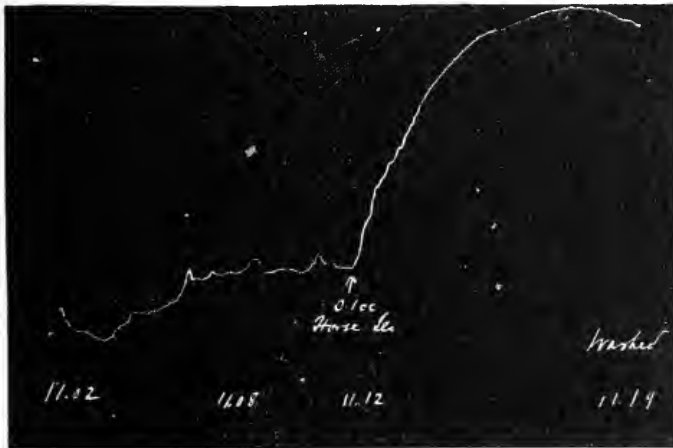
Toward the end of the decline, the addition of 0.3 cc. of horse serum produced no response. The cells were therefore desensitized to this antigen. But the subsequent addition of rabbit serum to which this animal had also been sensitized, provoked a sharp contraction. The muscle was therefore not paralyzed, but retained its normal anaphylactic reactivity.

Figures 3 and 4 are curves produced by opposite horns of the same uterus. In figure 3, the addition of 0.1 cc. horse serum in the usual manner produced a typical sharp response. Figure 4 presents a curve in some respects similar to figure 1. The striking irregularities of the curve remind one of the alternate recov-





On January 6, actively sensitized both to horse serum and to rabbit serum. Level of the drum unchanged throughout.



Sensitized on December 21 by the intraperitoneal injection of 0.1 cc. of horse serum. Tracing of the opposite horn of figure 4. The organ was kept in the Dale apparatus during the time occupied by the tracing shown in figure 4. It remained throughout in a condition of slightly varying tonus. At the end of the period, it responded typically to 0.1 cc. of horse serum.

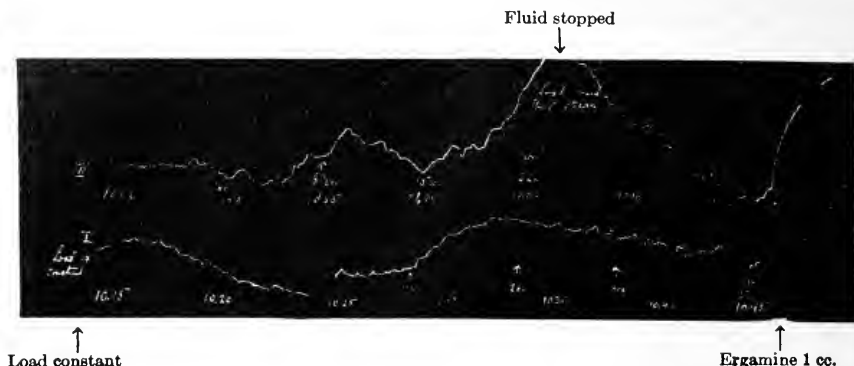


FIG. 4. GUINEA PIG 415

The figure records first a short preliminary tracing which shows the tendency of the muscle to undergo gradual relaxation. At 10.25 the delivery of 1 per cent horse serum from the burette was begun. With the transition from Line I to Line II, the level of the drum was changed, so that the base line of II is almost an inch above that of I.

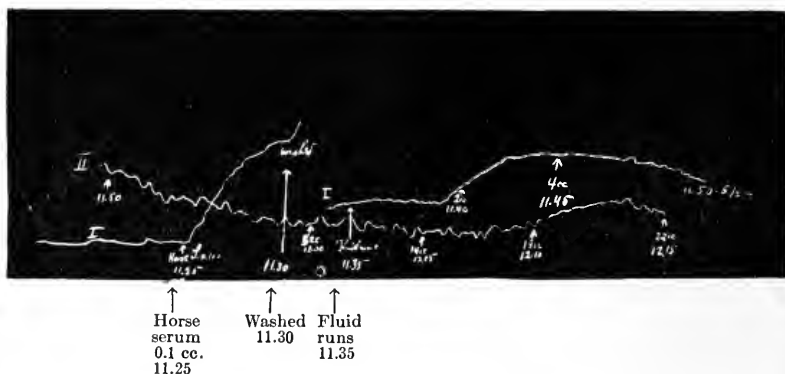


FIG. 5. GUINEA PIG 418

The opposite horn of uterus shown in figure 1. The preliminary tracing shows the response to 0.1 cc. of horse serum. This has served to desensitize the organ. The remainder of the curve shows that the gradual addition of horse serum failed to produce an anaphylactic response. The level of the drum was not changed in passing from Line I to Line II; the fact that the latter ends below the level of the former indicates a loss of muscle tonus.

eries and exacerbations manifested by the living animal in delayed shock. After "recovery," which would probably not have occurred with a reaction of corresponding severity in the living animal, the organ responds to ergamine.

Figure 5 serves as a control to the preceding tracings. It was traced by the opposite horn of the uterus shown in figure 1. The organ was first desensitized by 0.1 cc. of horse serum. After washing, the diluted horse serum was added from the burette up to 22 cc. Not only was there no appreciable contraction of the muscle, but, on the contrary, the tracing indicates a gradual and almost steady relaxation of muscular tone.

The sensitized uteri did not invariably respond to the gradual addition of the antigenic serum, but in some instances were apparently unaffected thereby. The normal controls regularly failed, under satisfactory experimental conditions to respond.

These experiments show that the isolated sensitized uterus reacts to the gradual addition of antigen in a fashion apparently quite analogous to delayed shock in the living animal. This fact, taken in conjunction with the preceding set of experiments, seems to justify the belief that delayed shock is caused by the reaction of cellular antibody with introduced antigen under certain special conditions. Free or circulating, antibody plays no part in its production. The factors in the mechanism of delayed shock are therefore exactly the same as in acute shock. The only difference consists in the fact that the union of cellular antibody with antigen in the one case is gradual, in the other, rapid and sudden. The question naturally arises why the cells are not desensitized, so as to fail to respond at all, by the gradually increasing concentration of antigen. In a previous study, (Study VI) (6), it was shown that the condition of desensitization is purely relative. Desensitized animals or uteri are still responsive to the sudden introduction of a very large dose of antigen. The present study indicates that desensitization modifies the response toward relatively enormous amounts of antigen when the concentration of the latter is steadily and gradually increased. The fact that any reaction whatever should occur under such conditions is an interesting amplification of our knowledge of cellular physiology in im-

munity. It is not improbable that this fact contains the explanation of some of the symptoms of infectious disease.

#### CONCLUSIONS

##### *Experimental observations*

1. Guinea pigs that have been actively sensitized several (6-17) months previously, and in which the circulating blood has, in addition been largely replaced by normal blood, present a delayed reaction upon the intraperitoneal injection of antigen. Therefore, cellular antibody mediates delayed shock.

2. The simultaneous injection of antibody intravenously, and of antigen intraperitoneally, does not induce any anaphylactic symptoms. Therefore, circulating antibody is not a factor in the production of delayed shock.

3. The isolated uterus effectively reproduces the picture of delayed shock, if the antigen is very gradually added from a burette.

##### *Theoretical deductions*

4. The factors in the mechanism of acute and of delayed shock are identical, namely the reaction of cellular antibody with freshly introduced antigen.

5. In the case of acute shock, the reaction is immediate, owing to the sudden introduction of antigen, which is, as a rule, accomplished by the intravascular route. In the case of delayed shock, the reaction is delayed, owing to the cumulative effect of the gradual absorption of antigen, as from the peritoneal cavity.

6. It is suggested that the gradual mode of reaction of the cells may possibly explain some of the symptoms of the infectious diseases.

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## STUDIES IN ANAPHYLAXIS

### XIX. SIMULTANEOUS INJECTIONS OF ANTIGEN AND ANTISERUM. THE ANAPHYLATOXIN THEORY OF ANAPHYLAXIS

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The investigation of the effects of simultaneous intravenous injections of antigen and antibody occupies one of the most interesting and important chapters in anaphylaxis. About ten years ago Otto (1) first showed that passive sensitization is invariably accompanied by a latent period of several hours duration. He interpreted the latent interval simply as the period of time required by the cells to appropriate the introduced antibody, and drew the fundamental inference that the anaphylactic response could not take place except through the mediation of cellular antibody. This theory, which is quite in harmony with Besredka's views, was at first very generally accepted. The discovery of anaphylatoxin, however, gave rise to a rival and altogether irreconcilable theory of anaphylaxis. When it was found that the incubation of antigen with immune serum in the test tube gave rise to violent poisons, it seemed natural to infer that the same poisons would be produced when antigen was injected into the sensitized animals. If, however, the inference from the test tube to the vital reaction was to be maintained intact, this new interpretation implied that the reaction in the living animal must take place in the circulating blood, and not in the cells. Thus originated the humoral theory of anaphylaxis. The chief objection to this latter conception is the latent period of passive sensitization. It is difficult to understand why the injection of the antiserum should necessarily be followed

by an interval of several hours before the animal becomes sensitized to the subsequent injection of the antigen. According to the anaphylatoxin theory the simultaneous injection of these two factors into the blood should at once provide the conditions requisite for their interaction, and thus for the production of anaphylactic shock. In order to vindicate the humoral theory, therefore, it became necessary to prove that simultaneous injections actually were effective in producing shock, and that the latent period is actually not a constant feature of passive sensitization. A great deal of study has been expended upon this problem, and it will be the object of the present paper not only to analyze the results of the experiments recorded by others, but also to contribute further experimental data along the same lines. Owing to the fact that conditions appear to differ in guinea pigs, rabbits, and dogs, the three species will be separately considered. Moreover, for purposes of convenience, it will be best to discuss first the effects of separate simultaneous injections, and then the effects of the incubated mixtures of antigen and antibody.

## 1. SIMULTANEOUS INJECTIONS

### *Guinea pigs*

Otto's demonstration of the latent period in guinea pigs was soon confirmed by Rosenau and Anderson, and by many others. The earlier workers, however, employed the intraperitoneal route for the sensitizing injection, and the possibility remained that part, if not all, of the latent interval was attributable to the delay in absorption. The introduction of the intravenous route eliminated this difficulty, and Doerr and Russ (2) repeated Otto's experiment, giving both the sensitizing and the intoxicating injections intravenously. Under these conditions they found that there is still a latent interval of at least three hours. Doerr and Russ maintained that the latent interval was a characteristic and almost constant feature of passive sensitization.

Doerr (3) states that it is, as a rule, not possible to induce shock if antigen and antibody are separately and simultaneously

injected into the veins, or if these substances are injected immediately after being mixed *in vitro*. This conclusion was confirmed by an experiment reported by the writer (4), in which antigen and antibody in varying ratios were injected simultaneously into the veins of guinea pigs.

Gurd (5), however, has disputed these results. This author employed as antibody the serum of rabbits immunized against sheep serum, and as antigen, sheep serum. The guinea pigs were injected with immune rabbit serum into one jugular vein, and immediately thereafter with sheep serum into the same or the opposite jugular vein. Analysis of the detailed protocols shows that seven of the eighteen animals so treated died, although in none did death occur in less than one hour after injection. In many of the animals that survived there were definite and pronounced symptoms. Gurd therefore concludes that "a period of incubation is not necessary in order that animals may become passively hypersensitive."

In considering these experiments by Gurd one is struck by the remarkable fact that he has apparently failed to include any controls, inasmuch as he did not give a separate *intravenous* injection of the immune serum to any of his animals. He did, indeed, give *intraperitoneal* injections of the immune serum without producing symptoms, but the two procedures are in no way comparable. It is well known, through the work of Doerr and Russ, Friedberger, and others, that the serum of rabbits immunized against sheep erythrocytes is highly toxic for guinea pigs, and that it may be immediately fatal when given intravenously in amounts as small as 0.1 cc., whereas the intraperitoneal injection of more than ten times this quantity will give rise to no symptoms whatever. Friedberger (6) has found that the serum of rabbits immunized to sheep serum may, likewise, be extremely toxic for guinea pigs. Thus, one such serum caused death with acute anaphylactic symptoms in amounts of 0.25 cc. given intravenously, although the intraperitoneal injection of very much larger amounts was well borne.

In view of the fact that of all those who have performed this type of experiment, Gurd alone has been able to produce anaphy-

lactiform symptoms with any degree of frequency, it is safe to assume that his results are to be attributed to the use of a highly toxic antiserum. Under the circumstances, therefore, his experiments cannot be considered as having any bearing upon the problem at issue.

There is, however, one point which requires further elucidation. Doerr and Russ occasionally produced symptoms by simultaneous injections of antigen and antibody, as did also Biedl and Kraus (7). Such results are, as Doerr (8) states, rare exceptions, and the symptoms so induced are generally mild. Zinsser (9) confirms this contention in the following words: "It is extremely difficult to obtain responses in guinea pigs or rabbits when the interval between the injection of the two is not observed. Personally, I have had distinct symptoms when injecting antigen and antibody together." In spite of their irregularity, these results have been obtained with sufficient frequency to require an explanation, and all of the three writers above mentioned have expressed the belief that the symptoms were produced by the interaction of antigen with antibody in the circulating blood. This interpretation leads inevitably to the view that anaphylaxis is, in part, even if only in lesser part, a humoral reaction.

There is, however, one serious defect in the preceding experiments on simultaneous injections, namely the lack of any controls. Such controls should obviously consist in the simultaneous intravenous injection of two alien sera which are not related as antigen and antibody. If severe symptoms are even occasionally produced, it is clear that some other factor must be invoked for their explanation than the immune mechanism, inasmuch as the latter is excluded by the terms of the experiment. Such a result would at once alter the significance of those experiments in which antigen and antibody have been simultaneously injected. As will be seen from the following protocols, the controls have actually given results of exactly this description. The negative experiments are not included, but it is as well to state that they constituted an even smaller proportion of the total than do the negative results of the simultaneous



injection of antigen and antiserum as reported by Doerr and others.

*Experiment 1.* In this experiment two sera were used, namely (1) the serum of rabbit 558 which had been immunized against cow's milk,<sup>1</sup> and (2) normal sheep serum. Preliminary tests showed that 2 cc. of the former and 1 cc. of the latter, when given alone, produced no symptoms when given by intravenous injection (guinea pigs 1, 2 and 3). In the second place, it was found that the former neither precipitated the latter in the test tube nor sensitized guinea pigs passively thereto (guinea pig 3). Finally, it was shown that their simultaneous injection intravenously in considerably smaller amounts than used in the controls gave rise to severe symptoms, and might even cause death.

1. Guinea pig 39. 1 cc. sheep serum i.v. (intravenously). No symptoms.

2. Guinea pig 695. 1 cc. sheep serum i.v. No symptoms.

3. Guinea pig 696. 2 cc. serum 558 i.v. No symptoms. On following day, 0.3 cc. sheep serum i.v. No symptoms. After an interval of 40 minutes, 0.4 cc. milk. Anaphylactic death.

4. Guinea pig 549. Simultaneous intravenous injection of 1 cc. S. 558, and 0.25 cc. sheep serum. No immediate symptoms. After 3 hours, pronounced weakness, inability to stand, prostration. Recovery after 5 hours.

5. Guinea pig 43. The same doses as in the preceding animal with a similar result.

6. Guinea pig 905. The same doses as in the preceding animal. No symptoms until  $1\frac{1}{2}$  hours; then increasing weakness and death in  $5\frac{1}{2}$  hours. Temperature 1 hour before death,  $96^{\circ}$ .

*Experiment 2.* The sera used were (1) the serum of rabbit 871 immunized against ox bile, and (2) normal sheep serum. No additional controls for the sheep serum will be cited.

1. Guinea pig 990. 2.3 cc. serum 871 i.v. Mild symptoms for 3 hours.

2. Guinea pig 694. Simultaneous i.v. injections of 1.5 cc. serum 871, 0.3 cc. sheep serum. After 1 hour, onset of weakness; after 1 hour and 40 minutes, convulsions, followed by apparent recovery. Dead next morning.

<sup>1</sup> It is perhaps worthy of note that symptoms are more frequently produced in this type of experiment when one of the two sera injected is an immune serum, although not specifically related to the other.

*Experiment 3.* In this experiment normal rabbit serum and sheep serum were the sera used. There are no additional controls for the sheep serum.

1. Guinea pig 504. 2.5 cc. normal rabbit serum i.v. No symptoms.
2. Guinea pig 502. Simultaneous i.v. injections of 1 cc. normal rabbit serum, 0.3 cc. sheep serum. No symptoms.
3. Guinea pig 503. Simultaneous i.v. injections of 1.5 cc. normal rabbit serum, 0.3 cc. sheep serum. After 15 minutes, severe prostration lasting for 1 hour. Recovery.

*Experiment 4.* The sera used were horse serum and sheep serum.

1. Guinea pig 691. 3.5 cc. horse serum i.v. No symptoms.
2. Guinea pig 968. Simultaneous i.v. injections of 1 cc. horse serum, 0.25 cc. sheep serum. After  $3\frac{1}{2}$  hours marked weakness and prostration. Recovery.

It appears from the experiments that the simultaneous intravenous injection of two different sera, not related as antigen and antibody, not infrequently gives rise to severe, or fatal symptoms. In all respects these symptoms are strikingly similar to those described by Gurd, and by others, particularly with regard to the delay in their onset. Such being the case, one cannot attribute any particular significance to those experiments in which the injected sera were specifically related as antigen and antibody. The effect of mixed injections evidently depends upon some general and unspecific effect, and not upon an immunological reaction. Thus the latter experiments lose all relation with the phenomena of anaphylaxis, and do not, as had been maintained, either establish, or even support, the humoral theory. They simply illustrate the effects of the injection of alien proteins into the circulation in guinea pigs.

The nature of this reaction, produced in guinea pigs by such injections of alien proteins, is quite unknown, but it is vaguely ascribed to a disturbance of colloidal equilibrium. It is well known that certain normal sera, when given intravenously to guinea pigs, even in relatively small amounts, produce toxic, or fatal symptoms. Such, for example are the sera of man, of the dog, and of the ox. The analogy of this fact is found in Dale's observation that normal sera induce a sharp contraction in the

suspended uterus of unsensitized guinea pigs. It is, therefore, not surprising that a mixture of two different sera should produce the same effect in heightened degree.

This belief is well borne out by the fact that those who have described results of this type have regularly injected relatively enormous amounts of antibody and antigen. An active rabbit-anti-horse serum will sensitize guinea pigs passively in amounts of 0.1 cc., so that the subsequent injection of 0.05 cc. of horse serum produces immediate anaphylactic death. The simultaneous injection of these two factors in such small amounts, however, is absolutely ineffective. Only with much larger quantities, such as 1 or 2 cc., are striking effects produced. Yet, if the mechanism is immunological it is reasonable to demand that doses similar to those effective in the typical experiment of passive anaphylaxis should be productive of symptoms.

It is interesting to note that the simultaneous injections of two sera related as antigen and antibody, and not their mere presence together in the blood of the guinea pig, appears to be the essential factor in the production of a reaction. Thus Biedl and Kraus discovered that they could not induce "anaphylactic" symptoms if even a very brief interval, such as ten minutes, were allowed to intervene between the injection of the antigen and the antiserum. Evidently this fact cannot be reconciled with the view that these two substances produce the symptoms through their interaction in the blood; ten minutes after injection it is certain that the major part of either would remain in the blood, in condition to react with the other. On the other hand, if disturbance of the colloidal equilibrium is the essential factor, it seems equally evident that this might be more violently accomplished by simultaneous, than by successive injections.

Thus far consideration has been limited to the injection of two different sera simultaneously into guinea pigs. Thiele and Embleton (10) have described experiments in which the simultaneous injection of alien red blood cells and the corresponding hemolytic serum produced acute anaphylactic death. Unfortun-

nately their experiments are complicated by the fact that they made use of the serum of rabbits immunized against sheep red blood cells, which, as has been stated, is well known to be highly toxic for guinea pigs. In my own experiments it has not been possible to inject guinea pigs intravenously with a passively sensitizing dose of this serum without causing immediate death. Doerr (11) likewise finds it difficult to harmonize Thiele and Embleton's results with his own experience with this type of antiserum. Aside from this objection, it is clear that this experiment is interpretable in terms of those previously described. The injection of these two reagents would be promptly followed by hemolysis of the sheep erythrocytes, thus throwing into the circulation almost simultaneously not only the rabbit antiserum, but also the dissolved proteins of the red cells. The details of the various experiments recorded by Thiele and Embleton seem actually to force this interpretation. When sensitized red cells alone were injected into normal pigs no reaction of any kind resulted. Now it is clear that if the hemolysis of the cells had been the cause of the immediate reaction, it should certainly have developed after the injection of the sensitized cells. Evidently it is not the hemolysis *per se* which affects the reaction, but the simultaneous freeing of two foreign proteins, rabbit antiserum and the dissolved protein of the sheep red cells, in the circulation of the guinea pig. It is evidence of the same fact that the authors failed to induce symptoms when the sheep red cells were given at an interval of fifteen minutes or more after the rabbit immune serum, instead of simultaneously. Unquestionably hemolysis would occur under these circumstances, but the interval would serve to protect the animal from the simultaneous liberation of two alien proteins in the circulation. This observation is directly analogous to that previously described by Biedl and Kraus with sera.

In order to exercise a proper control over these experiments, it would be necessary to inject guinea pigs simultaneously with the serum of a rabbit immunized against sheep cells, and with hemolyzed or sensitized red cells of some other species. The primary toxicity of the antiserum has made it impossible to carry

out this program. It has been possible, however, to give simultaneous injections of other serum together with hemolyzed red cells. The results of these experiments, although not constant, have indicated that such mixtures occasionally possess a degree of toxicity considerably greater than that represented by the summation of the toxicities of the two components. One group of such experiments is here detailed.

In the following experiment the injection material consisted of (1) normal rabbit serum and of (2) a 10 per cent solution of ox red cells in distilled water, which was then given the concentration of normal saline by the addition of one-tenth the volume of 8 per cent salt solution. In the controls, these fluids were injected separately, and in the test animals simultaneously. All the injections were intravenous.

#### *Controls*

Guinea pig 194 (195 grams).

3.30 p.m. 2 cc. 10 per cent ox cells; no symptoms.

3.50 p.m. 1 cc. n.r.s. (normal rabbit serum) mild symptoms.

Guinea pig 195 (265 grams).

3.45 p.m. 2 cc. n.r.s.; no symptoms.

4.25 p.m. 3 cc. 10 per cent ox cells; no symptoms.

#### *Positive results*

Guinea pig 189 (180 grams).

3.25 p.m. 2 cc. 10 per cent ox cells } together.  
0.5 cc. n.r.s.

Convulsions; death in three minutes.

Guinea pig 191 (240 grams).

3.38 p.m. 2 cc. 10 per cent ox cells } together.  
0.5 cc. n.r.s.

Immediate death.

#### *Negative results*

Guinea pig 182 (255 grams).

3.15 p.m. 1 cc. 10 per cent ox cells } together.  
2 cc. n.r.s.

No symptoms.

Guinea pig 197 (245 grams).

4.00 p.m. 2 cc. 10 per cent ox cells } together.  
1 cc. n.r.s. }

No symptoms.

Guinea pig 199 (240 grams).

4.35 p.m. 2 cc. 10 per cent ox cells } together.  
0.5 cc. n.r.s. }

No symptoms.

Thus it seems that the effects of simultaneous injections of hemolysin and of red blood cells in guinea pigs are erroneously attributed to their interaction in the blood. As a matter of fact, they produce the same effects as do two entirely unrelated proteins similarly injected.

The effects of the injection into guinea pigs of incubated mixtures of antigen and antibody, i.e., of anaphylatoxin, will be subsequently considered.

### *Rabbits*

Although the guinea pig has been made the subject of the great majority of experiments, the rabbit has also been similarly employed to a limited extent. Those who have reported experiments with the latter species are Briot (12), Scott (13), and Friedemann (14).

Briot is generally misquoted in this connection on account of an ambiguous statement in his original paper. In the errata of the same volume, however, he states specifically that the injections were given a day apart.

Scott states that a dose of 5 or 10 cc. of the serum of an immune rabbit "*immediately* confers on a fresh rabbit the capacity to react with the typical symptoms on injections with the appropriate proteid. As a rule, however, the symptoms are distinctly milder; they certainly are more transient." No experimental data are given in illustration of this statement.

Friedemann has made a more elaborate argument than any other author for the anaphylactic effects of simultaneous injections in rabbits. He drew a sharp contrast between the effects

of passive sensitization as seen in rabbits and in guinea pigs, maintaining that the former were hypersensitive for only a very short period immediately following the injection of immune serum, and that by the following day they had lost the capacity to react with antigen. In other words, he asserted that the humoral mechanism alone was effective in rabbits, although in guinea pigs the cellular antibodies were conceded to take part in the reaction.<sup>2</sup> The experiments of Friedberger, and of Pick (15), however, have shown clearly that Friedemann was in error on this point. When properly sensitized, rabbits react with characteristic symptoms upon the injection of the antigen on the following day. Indeed, Scott, as previously cited, found that the symptoms produced by simultaneous, were distinctly less marked than those produced by spaced injections.

## 2. INCUBATED MIXTURES. ANAPHYLATOXINS

It remains to consider Friedemann's claim, which has generally been admitted by those who accept the humoral theory, that his experiments demonstrate the effectiveness of simultaneous injections. As a matter of fact, in his entire and extensive series of protocols Friedemann includes no experiment which would support such a claim. In no instance were the injections of antigen and antibody made simultaneously and separately; the two substances were invariably mixed in the test tube, and were allowed to stand for a variable period of time before being injected. For this reason Friedemann in his conclusions (p. 636) states that passive sensitization is best demonstrated by means of the intravenous injection of a *mixture* of the two factors. When red blood cells constituted the antigen, Friedemann incubated the mixtures, and injected the superna-

<sup>2</sup> "The passive transfer of hypersensitiveness to a foreign serum by intravenous injection is most successful when the antigen and antibody are mixed before injection. If the antiserum is injected on the previous day, the symptoms are indefinite. A preliminary sensitization of the body cells by the antiserum, as in the case of the Theobald Smith phenomenon (in guinea pigs), is not borne out by the facts. It seems much more probable that the anaphylactic poison is formed within the blood stream." L. c., p. 636.

tant fluid obtained after sedimentation of the cells. When this experiment was successfully performed, as a rule no hemolysis whatever took place; nevertheless, the injection of the supernatant fluid proved extremely toxic to the rabbits. In view of the more recent studies of Bordet (16), of Nathan, and of Jobling (17), the significance of this experiment of Friedemann's has become quite clear. Immune serum, when mixed with its antigen, undergoes a profound chemical change due to autodigestion. The mechanism of this change is not perfectly clear; Jobling suggests that it is due to the adsorption of anti-ferment by the antigen-antibody complex. At all events, the chemical alterations are of so definite a character as to be easily demonstrable by quantitative methods of analysis. Exactly the same changes take place whether the antigen be serum, or cellular material such as erythrocytes or bacteria. In those of his experiments in which serum constituted the antigen, Friedemann gives no definite data as to the time relations; he does, however, state that precipitation occurred in these mixtures previous to their injection, from which it is evident that they were certainly not injected at the moment of preparation. The experiments of Nathan show that the chemical changes in serum previously referred to may take place within fifteen minutes, so that it is justifiable to conclude that there was ample time for their production in the serum mixtures given by Friedemann.

The bearing of these facts upon Friedemann's experiments is as follows: Friedemann concluded that the toxicity of these mixtures of antigen and antibody was nothing more nor less than the expression of those changes which are produced in the blood of sensitized animals upon the injection of antigen. The mechanism of anaphylaxis is, on this theory, reducible to the production of toxic substances in the circulation, as a result of the interaction of antigen and antibody in the blood. So evident did this inference appear that Friedberger coined the word anaphylatoxin to designate this hypothetical poison, whether formed in the test tube or in the sensitized animal, and, despite occasional protests, the term has gained almost universal currency. Even those who within recent years have shown that this toxin, as



produced *in vitro*, is derived from the immune serum, and not from the antigen as at first believed, still adhere to the view that the same processes occur in the living animal as in the test tube.

Attractive as is this theory on account of its apparent simplicity, it cannot be brought into any sort of harmony with the facts of anaphylaxis as at present understood. Unquestionably the incubation of antigen and antibody *in vitro* results in a toxic alteration of the mixture. But, just as unquestionably, the injection of these two substances separately and simultaneously into guinea pigs or rabbits is without marked effect in the living animal. It has been shown in the foregoing that the experiments of Gurd and of Thiele and Embleton, which alone seriously challenge this statement, are based upon the use of sera of very high primary toxicity for guinea pigs, namely that of rabbit versus sheep serum or versus sheep erythrocytes, a fact which at once invalidates their conclusions. On the other hand, there are the practically unanimous statements of all those observers who have not committed this error, including Doerr and Russ, Scott, and Zinsser, that the simultaneous injection of antigen and antibody does not produce anaphylactic death. At most, these observers have described mild, indefinite, and inconsistent results, such as are readily attributable, as shown in the preceding pages, to the disturbing effects of foreign proteins in the circulation. These observations seem to force the conclusion that such processes as take place in the test tube are actually without a true parallel in the living body. This conclusion is, indeed, very strikingly borne out by the experiments which have been made with agar. If agar is incubated with normal guinea pig serum, the mixture becomes highly toxic for guinea pigs; anaphylatoxin is said to be produced in large amount. Bordet's experiments have shown that the production of this anaphylatoxin is characterized chemically by exactly the same alterations as occur when an immune serum is incubated with its antigen (Jobling). On the other hand, when agar is injected intravenously into the living guinea pig, no symptoms whatever are produced. In the same fashion, Friedberger and

Nathan produced a highly effective anaphylatoxin by incubating normal horse serum with normal guinea pig serum. Yet the injection of horse serum into normal guinea pigs in any quantity which the cardio-vascular apparatus will permit is without effect. If further proof were necessary that serum is quite different from the circulating plasma, it is furnished by the fact that freshly drawn guinea pig serum, by itself, may prove highly toxic to the living animal. The contention of Friedemann, Friedberger, and many others, who have assumed that the changes that occur in serum when incubated with various substances such as agar, starch, or other serum, in the test tube, also occur when those same substances are injected into the living animal, are thus shown to be entirely at variance with all the facts as experimentally demonstrated. The two sets of conditions are found to be utterly different; what occurs in the test tube is due to special circumstances, which are lacking in the living animal.

Thus Friedemann's observations, and the long series of experiments upon anaphylatoxin as produced in the test tube, have no direct application to the conditions that obtain in the living animal. Neither direct experiment, nor correct inference, permit the belief that the symptoms of anaphylaxis can ever be induced by the interaction of antigen and antibody in the circulation of the living animal.

### *Dogs*

The data available in this species are so inadequate as to render a definite conclusion impossible. Both Pearce and Manwaring agree that transfusion experiments indicate the participation of a cellular antibody. Manwaring, however, seems to believe that in some dogs, though not in all, the humoral reaction may also play a rôle.

## CONCLUSIONS

*Experimental Observations*

1. In guinea pigs, characteristic anaphylactic symptoms have been produced only exceptionally by the simultaneous, but separate, injection of antigen and antibody intravenously. Where highly toxic antisera were unwittingly used for this experiment, as by Gurd and by Thiele and Embleton, the experimental conditions nullify the conclusions.

2. Anaphylactiform symptoms and death may be produced in guinea pigs by the simultaneous separate injection of two foreign sera or proteins not related as antigen and antibody. This result is evidently due to the generalized, non-specific, effect of foreign proteins on the organism; it explains the exceptional instances of reaction mentioned under 1, which are therefore erroneously attributed to an immunological mechanism.

3. In rabbits, characteristic anaphylactic symptoms have never been induced by separate, simultaneous injections.

4. The injection of previously prepared mixtures of antigen and antibody frequently produces violent anaphylactiform symptoms in guinea pigs and in rabbits. This is due to certain chemical alterations ("anaphylatoxins") which may also be produced by incubating normal serum in vitro with a great variety of substances, such as agar, starch, heterologous serum, etc.

5. The injection of these latter substances intravenously has never been shown to produce anaphylactic symptoms. It appears, therefore, that serum in the test tube reacts quite differently from plasma of the circulating blood.

*Theoretical Deductions*

6. The interaction of antigen and of antibody in the circulating blood does not give rise to toxic substances (anaphylatoxins) and does not produce anaphylactic symptoms.

7. Anaphylaxis, by which is meant the reaction of the previously treated animal to a fresh injection of antigen, is always and invariably mediated by cellular antibody, and by that alone.

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## STUDIES ON ANTIBODIES. I

### ANALYSES AND NITROGEN DISTRIBUTION OF A NUMBER OF ANTISERA

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The antisera analyzed were the following:

Antidiphtheria globulin, 2900 units per cubic centimeter.  
Designated A in the tables.

Antidiphtheria globulin, 975 units per cubic centimeter.  
Designated H.

Antitetanus globulin, 1900 units per cubic centimeter. Designated B.

Antitetanus serum, 500 units per cubic centimeter. Designated F.

Antitetanus serum, 60 units per cubic centimeter. Designated G.

Antistreptococcus serum. Designated C.

Antigonococcus serum. Designated I.

Antimeningococcus serum. Designated E.

Normal serum. Designated D.

The method of separating and concentrating the diphtheria and tetanus antitoxin together with the pseudoglobulin with which they are associated was carried out as follows:

The plasma from the immunized horse was diluted with an equal volume of water and heated to 61°C. An equal volume of saturated solution of ammonium sulphate was then added. The resulting precipitate contained the fibrinogen, euglobulin, pseudoglobulin and antitoxin. This precipitate was taken up with water and reprecipitated with an equal volume of saturated solution of ammonium sulphate to free it from traces of albumin.

After draining the precipitate, it was taken up in water. In order to separate the fibrinogen and euglobulin from the pseudoglobulin and antitoxin, the mixture was saturated with sodium chloride. The resulting precipitate was filtered out. To the clear filtrate containing only pseudoglobulin and antitoxin, acetic acid was added to excess. The precipitated pseudoglobulin and antitoxin were recovered on hardened filter paper, pressed and dialyzed.

Antidiphtheria globulin designated in the tables "A" was prepared from the bleedings of one horse. Antidiphtheria globulin "H" was prepared from bleedings from nine horses.

The antitetanus globulin "B" was prepared from the bleedings from two horses.

The antitetanus serum "G" was taken from a horse that had been bled nineteen times.

The antitetanus serum "F" was taken from a horse that had been bled six times.

The antistreptococcus serum "C" represents the sixty-sixth bleeding from a horse.

The antigenococcus serum "I" represents the fourth bleeding from a horse.

The antimeningococcus serum "E" represents the twelfth bleeding from a horse.

The normal serum "D" represents the first bleeding from a horse.

The various antisera were placed in shallow pans and dried in a vacuum at 35°C., scaled from the pans and kept in a vacuum desiccator over calcium chloride.

The hydrogen ion concentrations of these substances in aqueous solution were determined with 0.1 gram in 15 cc. water by comparison of the color changes with various indicators with the standard solutions suggested by A. A. Noyes.<sup>1</sup> The results are given in table 1.

The methods of analysis were in general those commonly employed. They will be described briefly.

<sup>1</sup> Jour. Amer. Chem. Soc., 1910, **32**, 823.

*Moisture.* The substance in a beaker or platinum dish was dried in an air bath at 100–105° for three to four hours and the loss in weight determined. The heating was repeated for one-hour periods until the weight had become constant. After heating, the substance was allowed to cool each time in a desiccator over calcium chloride.

*Ash.* The substance was carefully ignited in a platinum dish, finally heated with a Meeker burner after the addition of three to four drops of concentrated nitric acid. The heating was repeated until there was no further loss in weight. The color of the ash was white in every case.

TABLE I  
*Hydrogen ion concentrations of preparations in water*

SUBSTANCE	APPEARANCE OF MIXTURE	INDICATORS	HYDROGEN ION CONCENTRATIONS, H.
A	Partly dissolved.....	Rosolic acid } Cochineal }	–5.5
B	Almost all dissolved.....	Rosolic acid	–7.0
C	Almost all dissolved.....	Rosolic acid } Phenolphthalein }	–8.5
D	Clear solution.....	Phenolphthalein } Thymolphthalein }	–9.5
E	Partly dissolved.....	Phenolphthalein	–8.5
F	Partly dissolved.....	Phenolphthalein } Thymolphthalein }	–9.5
G	Partly dissolved.....	Phenolphthalein	–8.5
H	Partly dissolved.....	Rosolic acid	–6.5
I	Clear solution.....	Phenolphthalein } Thymolphthalein }	–9.8

$P_2O_5$ . The ash was dissolved in about 25 cc. of water and a little nitric acid with warming. To the solution 12 cc. of ammonium nitrate solution (750 grams  $NH_4NO_3$  dissolved in water and diluted to 1 liter) and 50 cc. of molybdate solution (60 grams  $MoO_3$ , 440 cc.  $H_2O$ , 60 cc.  $NH_4OH$ , sp. gr. 0.90, poured into a mixture of 250 cc.  $H_2O$  and 250 cc.  $HNO_3$  sp. gr. 1.40, filtered after standing several days) were added, the mixture heated at 80° on a water bath for ten minutes, cooled and allowed to stand overnight. It was then filtered on asbestos in a Gooch crucible,





washed five to six times with an  $\text{NH}_4\text{NO}_3$  solution (containing 50 grams of the salt and 10 cc. concentrated  $\text{HNO}_3$  in 1 liter), once with 1 per cent  $\text{HNO}_3$  solution, dried in an air oven at  $120^\circ$  and weighed as  $(\text{NH}_4)_3\text{PO}_4 \cdot 12\text{MoO}_3$ .

*Nitrogen.* The Kjeldahl method with sulfuric acid, potassium sulfate, and a small amount of copper sulfate was used. The ammonia was distilled into standard sulfuric acid which was then titrated with sodium hydroxide solution with alizarin sulfonic acid as indicator. The substance was dried in an air bath at  $101$  to  $104^\circ$  for four hours in every case.

Table 2 shows the experimental data obtained in the analyses. The headings of the columns are perhaps sufficient to indicate the meaning of figures.

The mean results of the analyses are given in table 3.

TABLE 3  
*Mean results of the analyses of antitoxin preparations*

SUBSTANCE	MOISTURE	ASH	$\text{P}_2\text{O}_5$	(DRIED MATERIAL) NITROGEN
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
A.....	3.44	0.63	0.13	13.78
B.....	2.94	0.80	0.14	14.07
C.....	3.57	1.95	0.22	13.73
D.....	3.32	2.27	0.33	13.10
E.....	3.63	1.84	0.22	13.39
F.....	4.78	1.54	0.16	13.94
G.....	4.80	1.97	0.28	13.40
H.....	5.27	0.87	0.11	13.79
I.....	5.58	2.19	0.26	13.36

Since the amount of moisture adhering to the solid preparations after grinding to a fine powder and drying in a vacuum desiccator over calcium chloride appears to be variable, it appears best to compare the composition of the preparations after correcting for the moisture content.<sup>2</sup> Table 4 shows the per-

<sup>2</sup> The fact that drying in vacuum desiccator over calcium chloride does not remove all the moisture was shown by Falk and Suguira (Jour. Amer. Chem. Soc. 1915, **37**, 219) for enzyme preparations analogous in composition. These results show that preparations dried as stated will lose moisture when placed in a vacuum desiccator over phosphorus pentoxide or if heated to  $100$ – $110^\circ$ . The loss by the last two methods was practically the same.

centage of nitrogen and of ash of the dried preparations in columns 2 and 3; of nitrogen in the dried and ash free preparations in column 4; and finally in column 5 are given the percentages of phosphorus pentoxide in the ash of the different preparations.

In studying the results given in table 4 some interesting facts are apparent.

1. The low percentage of ash in the moisture free globulins (A, B, H) is accounted for in the prolonged dialization of these globulins to free them from ammonium sulphate. After removal of ammonium sulphate 0.6 per cent to 0.8 per cent of so-

TABLE 4  
*Corrected results of analyses of preparations*

SUBSTANCE	DRIED PREPARATIONS		DRIED AND ASH FREE NITROGEN	PER CENT $P_2O_5$ OF ASH
	Nitrogen	Ash		
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
A.....	13.78	0.65	13.87	20.6
B.....	14.07	0.82	14.19	17.5
C.....	13.73	2.02	14.01	11.3
D.....	13.10	2.35	13.42	14.5
E.....	13.39	1.91	13.65	12.0
F.....	13.94	1.62	14.17	10.4
G.....	13.40	2.07	13.68	14.2
H.....	13.79	0.92	13.92	12.6
I.....	13.36	2.32	13.68	11.9

dium chloride is added to bring the globulins up to a near physiological sodium chloride content. For the two tetanus sera (F and G), the more active preparation (F) contained 1.62 per cent ash, the less active 2.02 per cent ash. The normal serum contained the highest percentage of ash.

2. For the nitrogen content of the moisture free preparations, the less active tetanus serum (G) contained less (13.40 per cent) than the more active (F, 13.94 per cent). The less active diphtheria globulin (H) contained the same percentage of nitrogen as the more active (A). The nitrogen contents for all of the preparations are considerably smaller than those recorded in the literature for the proteins of horse serum.

3. The  $P_2O_5$  contents of the ash ranged from 10 per cent to 20 per cent. The markedly higher values for the tetanus and more active diphtheria globulins are noteworthy. As for the rest, no very distinct differences appear except possibly the comparatively large value for the normal serum and the smaller value for the more active tetanus serum 10.4 per cent as compared with 14.2 per cent for the less active.

The different preparations were finely ground and extracted with dried ether in Soxhlet extractors for several days. The ether extracts on evaporation gave residues amounting to approximately 0.1 per cent of the material extracted. The analyses of the extracted substances showed no marked differences in comparison with those of the unextracted substances.

TABLE 5  
*Formal titrations of antitoxin preparations*

SUBSTANCE	TITRATION cc. 0.1 NaOH	MG. N AS NH <sub>2</sub> : COOH	PER CENT N AS NH <sub>2</sub> : COOH
A.....	0.40	0.56	0.56
B.....	0.26	0.36	0.36
C.....	0.35	0.49	0.49
D.....	0.31	0.43	0.43
E.....	0.25	0.35	0.35
F.....	0.29	0.41	0.41
G.....	0.44	0.62	0.62
H.....	0.59	0.83	0.83
I.....	0.64	0.90	0.90

A number of routine tests were performed with the preparations. Positive reactions were obtained for all with the Millon test for proteins,<sup>3</sup> the glyoxylic acid test for tryptophane,<sup>4</sup> the Folin test for tyrosine,<sup>5</sup> and the Molisch test for carbohydrates.<sup>6</sup>

At present there is no apparent regularity to be observed in these results.

Since these antitoxin preparations appeared to be essentially protein in character, an attempt was made to characterize more

<sup>3</sup> Cf. H. C. Sherman, *Methods of organic analysis*, 1912, p. 315.

<sup>4</sup> *Ibid.*, p. 315; also S. R. Benedict, *J. Biol. Chem.*, 1909, **6**, 51.

<sup>5</sup> *J. Biol. Chem.*, 1912, **12**, 239, 245.

<sup>6</sup> Sherman, *Loc. cit.*, p. 57.

particularly the different groups of amino acids which were present. The methods of analysis described recently by van Slyke were used.<sup>7</sup> A very concise summary of the principles underlying the separation is given by Percival Hartley<sup>8</sup> from whom the following description is quoted.

In carrying out the analyses of the different serum proteins the experimental conditions were kept as constant as possible. Two grams of material were boiled with 50 cc. of 20 per cent hydrochloric acid and the rate of hydrolysis was followed by testing samples of the fluid at intervals in van Slyke's apparatus. The hydrolysis was continued for sixteen to eighteen hours.

After hydrolysis of the protein the excess of hydrochloric acid was removed in vacuo and the hydrolyzed material washed into a 250 cc. flask. Duplicate quantities of 25 cc. of the solution were used for the determination of the total nitrogen, and 200 cc. of the solution were used in all cases for analysis. The sum of the ammonia nitrogen, melanin nitrogen, total nitrogen of the filtrate, and the total nitrogen of the bases (the last named being determined in two parts) should be the same as the amount originally present, as determined separately in the 25 cc. portions. . . .

. . . . In separating the diamino acids from the monoamino acids the same amount of phosphotungstic acid (15 grams) was used, and the precipitation was carried out at a volume of 200 cc. in all cases. After the addition of the phosphotungstic acid the mixture was heated on the water bath until most of the precipitate had redissolved and after cooling to room temperature the Erlemeyer flask was allowed to stand for forty-eight hours in the ice-box. The precipitate of the bases was washed with an ice-cold solution of phosphotungstic acid in hydrochloric acid, 120 to 140 cc. of the solution being used.

One of the more difficult of the estimations in the process is the determination of the total nitrogen of the bases, which is carried out

<sup>7</sup> The analysis of proteins by determination of the chemical groups characteristic of the different amino acids, *J. Biol. Chem.*, 1911, **10**, 15. The quantitative determination of aliphatic amino groups, *ibid.*, 1912, **12**, 275. The gasometric determination of aliphatic amino nitrogen in minute quantities, *ibid.*, 1913, **16**, 121.

<sup>8</sup> The determination of the composition of the different proteins of ox and horse serum by the method of Van Slyke, *Biochem J.*, 1914, **8**, 541.

on the same solution used for the determination of the arginine. In these analyses the practice was adopted of continuing the digestion for some time after the solution becomes clear (not colored) gently moving the fluid round the sides of the flask from time to time in order to wash down into the acid any particles which may have crept on to the upper parts of the digestion flask. The value of the histidine nitrogen is not determined directly but is calculated from the results of three other determinations, viz., the arginine nitrogen, the amino nitrogen of the bases, and the total nitrogen of the bases. As van Slyke points out these three values can all be determined accurately, and the duplicate analyses for the histidine should not vary by more than 1 per cent of the total nitrogen.

The following estimations were carried out on the solution of hydrolysed protein:

1. Total nitrogen was estimated in 25 cc. of the original solution.
2. Ammonia nitrogen and melanin nitrogen were estimated in 200 cc. of the original solution.
3. After the removal of the ammonia and melanin the separation with phosphotungstic acid was carried out.
4. The solution of the bases was made up to 50 cc. and the following estimations made:
  - (a) Cystine nitrogen in 10 cc.
  - (b) Amino nitrogen in 2 cc.
  - (c) Arginine nitrogen and total nitrogen of the bases in the same sample of 25 cc.
5. The filtrate from the phosphotungstic acid precipitate of the bases was made to 100 cc. and the following estimations made:
  - (a) Total nitrogen of the filtrate in 25 cc.
  - (b) Amino nitrogen of the filtrate in 2 cc.

Table 6 contains a record of the actual observations in the analyses, the volumes given in the headings referring in every case to the amount of the original solution corresponding to the volume actually employed as explained above. Three preparations were analyzed; normal serum (D), tetanus globulin (B), and the more active diphtheria globulin (A).

The results of the analyses given in table 6 are presented in table 7 as percentages of the nitrogen in the different forms in terms of the total nitrogen. The first row gives the results for normal serum, the second for the more active diphtheria globu-

TABLE 6  
*Details of observations*  
*Nitrogen distribution according to the method of Van Slyke*

MATERIAL	QUAN- TITY TAKEN	TOTAL N			AMMONIA		MELANIN		CYSTINE		ARGININE		TOTAL N OF BASES							
		N cc. $\frac{10}{10}$ acid per 25 cc.	g. N per 250 cc.	cc. $\frac{10}{10}$ acid per 250 cc.	N cc. $\frac{10}{10}$ acid per 250 cc.	g. N per 250 cc.	g. Ba SO <sub>4</sub> per 50 cc.	g. N per 250 cc.	cc. $\frac{10}{10}$ acid per 125 cc.	g. N per 250 cc.	cc. $\frac{10}{10}$ acid per 125 cc.	N cc. $\frac{10}{10}$ acid per 125 cc. Arginine titration	g. N per 250 cc.							
	grams																			
Normal (D).....	2	16.83	0.2357	10.09	0.0141	3.38	0.0047	0.0146	0.0044	3.96	0.0222	16.44	20.40	0.0572						
Tetanus glob- ulin (B).....	2	17.67	0.2475	13.03	0.0183	4.35	0.0061	0.0120	0.0036	3.69	0.0207	15.19	18.88	0.0529						
Diphtheria globulin (A).	2	18.01	0.2523	13.96	0.0196	5.01	0.0070	0.0137	0.0041	3.85	0.0216	15.16	19.01	0.0533						
MATERIAL	AMINO N OF BASES				AMINO N OF FILTRATE				TOTAL N OF FILTRATE				TOTAL N OF FILTRATE							
	cc. N per 10 cc.		g. N per 250 cc.		cc. N per 5 cc.		g. N per 250 cc.		cc. $\frac{10}{10}$ acid per 62.5 cc.		g. N per 250 cc.		Total N recovered per 250 cc.							
Normal (D).....	2.580; 18.3°; 764 mm.				0.0372				5.325; 20.5°; 754 mm.				0.1499				0.2380			
Tetanus glob- ulin (B).....	2.595; 24.0°; 759 mm.				0.0362				5.956; 25.0°; 758 mm.				0.1647				0.2521			
Diphtheria globulin (A).	2.559; 24.0°; 763 mm.				0.0359				5.894; 24.5°; 762 mm.				0.1644				0.2546			

lin, and the third for the tetanus globulin. In the next three rows are quoted some results from Hartley's paper for the whole protein of horse serum, and then for the euglobulin obtained from this serum by two methods, the ammonium sulfate method and Panum's method. Where two series of results are given, their mean was taken.

No very marked differences in the compositions of the different preparations appear from this table. At the present time, the only points to which attention may be called are the higher values for cystine nitrogen in the tetanus and diphtheria globulins compared with the globulins given by Hartley, although the normal whole proteins show differences in the same direction; that is, a higher value was found for the cystine nitrogen in normal serum in this work than was found by Hartley in total protein. The histidine nitrogen was, however, distinctly lower for the tetanus and the diphtheria globulins than for any other of the preparations. Also the non-amino nitrogen was slightly higher for them than for the other normal substances.

TABLE 7  
*Results of analyses expressed in percentages of total nitrogen*

MATERIAL	AMMONIA	MELANIN	CYSTINE	ARGININE	HISTIDINE	LYSINE	AMINO N. OF FIL- TRATE	NON-AMINO N. OF FILTRATE	TOTAL N. RECOV- ERED
Normal serum (D).	6.0	2.0	3.0	10.8	3.7	11.1	61.4	3.0	101.0
Tetanus-globulin (B).....	7.4	2.5	2.5	9.7	2.3	11.0	64.4	2.1	101.9
Diphtheria-globu- lin (A).....	7.7	2.8	3.3	9.8	2.0	9.1	64.2	2.1	101.0
Hartley's whole protein.....	7.3	1.6	2.1	9.5	5.6	12.8	60.0	2.0	100.9
Euglobulin (NH <sub>4</sub> )- SO <sub>4</sub> method.....	7.9	2.3	1.7	8.3	5.5	10.0	62.2	2.2	100.1
Euglobulin Pa- num's method....	8.0	2.3	1.8	9.4	5.2	10.1	61.0	1.8	99.7





# SCIENTIFIC PROCEEDINGS OF THE SOCIETY FOR SEROLOGY AND HEMATOLOGY, NEW YORK

October 6, 1916

## 1. INDIGO CHEMICAL TEST FOR SYPHILIS

*E. P. Flood:* See this issue, p. 69.

## 2. DIAGNOSIS AND SERUM TREATMENT OF ACUTE ANTERIOR POLIOMYELITIS

*Abraham Zingher:* Several methods have been suggested during the past summer in the treatment of anterior poliomyelitis. The one method that has given the most satisfactory results, has been the use of serum obtained from immune donors that have had poliomyelitis either recently or from one to several years previously. The serum was administered intraspinally in doses of from 10 to 15 cc. and repeated every twenty to twenty-four hours until two to three doses were injected. An attempt was made to obtain as large a supply of serum as possible and make it available to the members of the medical profession in this and adjoining states. The writer himself had the opportunity of using the serum in 160 cases at the Willard Parker Hospital and in 33 cases at the Minturn Hospital. In addition, the serum was supplied for 225 cases in the private practice of various physicians.

The serum injected intraspinally in the acute stages of poliomyelitis produces a moderate polynuclear leucocytosis, which is increased in intensity by the presence of hemoglobin and by the tricesol that was added as a preservative. This cellular reaction is not specific, since similar reactions were obtained with normal human serum, the secondary albumoses of Jobling, and to a less extent with horse serum. It is probable that the phagocytic action of the leucocytes is enhanced by the presence of specific antibodies in the immune serum. If some of the recent work of Rosenow is verified and the disease is found to be caused by the invasion of the vascular portions of the spinal cord and brain by an attenuated streptococcus producing lesions most probably of an embolic type, then the conceptions of the pathology and treatment of the disease will have to change. It is known, however, that one of the chief weapons of the body against the streptococcus is the phagocytic action of the polynuclear leucocytes.

The effect of the immune serum seemed to be fairly well shown by the 33 cases treated in the Minturn Hospital. These cases were

carefully observed and received the full treatment of two to three injections. Of the 33 cases 14 were in a preparalytic stage of the disease at the time the serum was administered; of the fourteen, eight remained free from paralysis, two developed paralysis within twelve to eighteen hours after the first dose of serum, and four developed paralysis forty eight hours or more after the injection of the serum. Of these four, two patients showed an involvement of the extremities, one of the right side of the face and one of an external rectus of the eye. The rapid and decided subsequent improvement in these cases was noticeable. None of the cases treated in a preparalytic stage of the disease died. Of the 19 cases treated with serum after paralysis had set in, 3 died soon after the injection (within twenty-four hours) and 16 recovered with varying degrees of motor impairment.

For purposes of studying the action of the immune sera, they were divided into various groups, depending upon the length of time which had elapsed since these donors had had their attack of infantile paralysis.

Early convalescent serum.....	2 to 6 months after the attack
Late convalescent serum.....	6 to 12 months after the attack
Group A serum.....	1 to 5 years after the attack
Group B serum.....	5 to 15 years after the attack
Group C serum.....	15 to 30 years after the attack
Group D serum.....	30 years or more after the attack

The value of the older sera is still on trial; it is possible that the value of such sera is purely that of normal serum, a rich protein fluid, which also causes a distinct polynuclear cell response in the spinal canal. A number of cases were therefore also treated with normal human serum obtained from healthy donors who had never, to their knowledge, had anterior poliomyelitis.

To test the value of immune serum in treatment it is important to select early cases in the preparalytic stage of the disease. The symptoms of meningeal irritation like stiffness of the neck, irregular muscular tremors and vomiting, should lead to the making of a lumbar puncture. The spinal fluid will generally show an increased number of leucocytes, mostly lymphocytes (from 20 to 500 or more), and a definitely increased amount of albumin and globulin. Even at the bedside the rich increase in cells can be recognized in these early cases by a characteristic macroscopic appearance in the spinal fluid, when it is examined in a test tube by transmitted light. A uniform ground-glass appearance of the fluid, caused by the increased number of suspended leucocytes, which appear as small floating dust-like particles, is quite a characteristic appearance, and will be found during an epidemic of diagnostic value. The increased amount of albumin can also be determined at the bedside by vigorously shaking a test tube half-filled with the spinal fluid. A high dense foam layer will appear on the surface, indicating the increased amount of albumin. A normal spinal fluid only shows a moderate foam after shaking, which soon disappears.

3. TECHNIC OF THE WASSERMANN REACTION: (a) THE DOSE OF COMPLEMENT; (b) THE DOSE OF ANTIGEN

*R. Ottenberg:* See this issue, p. 39 and p. 47.

4. A STUDY OF THE LAWS GOVERNING THE REACTION OF THE PARTIALLY DESENSITIZED CELLS

*Richard Weil:* It is a well established fact that the cell produces certain modifications in the properties of the antibodies which it anchors. Thus it has been shown that the reactivity of such antibodies is materially increased by the cell. The present report shows that partially saturated antibody, when anchored to the cell, also acts differently from antibody similarly treated when in suspension in serum. We may assume from previous work that the anaphylactic antibody and precipitin are identical, the former being anchored to the cell, the latter in circulation. It has been shown<sup>1</sup> that the precipitin combines quantitatively with the antigen.

The method of the present study was as follows: One series of guinea pigs was passively sensitized with the minimal sensitizing dose of the serum of a rabbit highly immunized against horse serum, another series against several multiples of this dose. For each of these series, the minimal anaphylactic dose was determined. Other series, passively sensitized with the same doses of the rabbit serum, received an immediate injection of the antigen in varying amounts, after which the anaphylactic dose was again determined in each series. A comparison of the results thus obtained revealed the fact that antibody does not react quantitatively with antigen. The minimal anaphylactic dose, after desensitization, can, under no circumstances, be explained as the result of neutralization of a certain fraction of the cellular antibody, leaving the balance free to react. There appears, rather, to be a general distribution of all antigen, no matter how much is introduced, over all antibody, producing an enormous diminution of the reactivity of the latter. There is thus a distinct difference in the relationship of cellular antibody to antigen, as compared with that of circulating antibody.

5. HAY FEVER. NATURE OF THE PROCESS AND THE MECHANISM OF THE ALLEVIATING EFFECTS OF SPECIFIC TREATMENT

*R. A. Cooke, E. P. Flood and A. F. Coca:* To be published in a succeeding number of this Journal.

<sup>1</sup>Weil R., Jour. of Immunology, 1, 1, 1.



# THE DISAPPEARANCE OF AGGLUTININ FROM THE BLOOD OF ANAPHYLACTIC AND NORMAL ANIMALS

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In order to throw light on the question of the origin of "anaphylatoxin," Doerr and Pick (Centralbl., 62, Orig., p 146) and Römer and Viereck (Zeitschrift f. Immunitätsforsch., Orig., Bd. xxi, p. 32) measured the disappearance of antibodies in heterologous sera in anaphylactic animals.

Doerr and Pick injected a rabbit sensitized to horse serum and a normal rabbit intravenously with cholera agglutinating horse serum, and they found that the amount of agglutinin and of the precipitable horse albumen remained the same in both for about a day and then disappeared faster from the anaphylactic animal than from the other. They injected guinea pigs sensitized to horse serum intraperitoneally with 1 to 2 cc. of cholera agglutinin from the horse and found that the agglutinin and the precipitable horse albumen appeared in the circulation of the sensitized animals more quickly than in that of the others and, up to six hours after injection, they were present in greater quantity. They conclude that a more rapid breaking down of antigen in anaphylactic animals than in normal ones is, at least in the first twenty-four hours, not indicated by their experiments.

Römer and Viereck injected guinea pigs sensitized to horse serum intracardially with diphtheria antitoxin from the horse and found that the antitoxin disappeared more quickly from their blood than from that of the controls.

Our experiments follow the work of these authors. It was thought desirable to eliminate the question of absorption and

to limit the problem to the destruction of agglutinin in a heterologous serum in animals sensitized to that serum and in normal animals. Therefore intracardiac and in two cases intravenous injections were resorted to. An agglutinin so strong that it could be detected in minute quantities was used for injection, and guinea pigs sensitized with a very small dose of serum were selected as the experimental animals.

*The experiments.* On May 6 a number of guinea pigs, weighing between 400 and 450 grams were sensitized with 0.004 cc. of rabbit serum given subcutaneously. May 27, twenty-one days later, one of these together with a control was given intracardially 0.2 cc. of coli-agglutinating rabbit serum with a titer of 1:60000. The sensitized animal scratched itself vigorously, had disпноea and lay on its side: that is, it had moderate anaphylaxis. The control did not react. Samples were taken from the hearts of both animals five minutes and two hours after injection. Tested in dilution with an emulsion of the strain used to produce the agglutinin the results were as follows:

AGGLUTININ-DILUTION	SENSITIZED ANIMAL		NORMAL ANIMAL	
	Five minutes	Two hours	Five minutes	Two hours
1 : 100	++	++	++	++
1 : 200	++	+	++	++
1 : 250	++	+	++	++
1 : 330	++	0	++	+
1 : 420	++		+	0
1 : 500	++		0	0

++ = complete agglutination.

+ = partial agglutination.

In other words the blood of the animal that had been sensitized nineteen days before showed an agglutinin content higher than that of the control five minutes after the reinjection, and a content lower than that of the control two hours later.

June 8, thirty-three days after the first injection, more reinjections were undertaken and it was found that the dose of 0.2 cc. of serum was too high—the animals all died of the shock. The dose was reduced to 0.1 cc. but even then some of the animals died. These were immediately bled by cutting the neck

veins. The injections were made on different days up to June 24 but as the animals behaved similarly at different times separate dates are not noted. Macroscopic technique was used. The results were read in ignorance of the identity of the samples, and they are noted in the succeeding tables according to the following scale.

- ++++ = agglutination complete and liquid clear.  
 +++ = agglutination complete and coagulum unsettled.  
 ++ = fine agglutination.  
 + = barely perceptible agglutination.

The first three animals with their controls received 0.2 cc. agglutinin.

*Intracardiac injections*

AGGLUTININ-DILUTION	DIED OF TYPICAL ANAPHYLACTIC SHOCK IN FIVE MINUTES	CONTROL, FIVE MINUTES
1 : 25	++++	++++
1 : 50	+++	++++
1 : 100	+	++++
1 : 200	0	+++
1 : 330		++
1 : 420		+
1 : 500		0
AGGLUTININ-DILUTION	DIED OF TYPICAL ANAPHYLACTIC SHOCK IN SEVEN MINUTES	CONTROL, SEVEN MINUTES
1 : 25	++++	++++
1 : 50	++	+++
1 : 100	0	++
1 : 200		+
1 : 250		0
AGGLUTININ-DILUTION	TYPICAL SHOCK, SEVERE REACTION SAMPLE, TWO HOURS	CONTROL, TWO HOURS
1 : 25	0	+
1 : 50	0	0

The following animals all received 0.1 cc. of agglutinin.

*Intravenous injection*

AGGLUTININ-DILUTION	DIED OF ACUTE SHOCK IN TWO MINUTES	
1 : 25	++++	
1 : 50	++	
1 : 100	0	

*Intracardiac injections*

AGGLUTININ-DILUTION	DIED IN THREE MINUTES	CONTROL, THREE MINUTES
1 : 25	++++	++++
1 : 50	+++	++++
1 : 100	+	++
1 : 200	0	0

AGGLUTININ-DILUTION	DIED IN FOUR MINUTES. SMALL ANIMAL	
1 : 25	++++	
1 : 50	++++	
1 : 100	+++	
1 : 200	+	
1 : 225	+	
1 : 330		

AGGLUTININ-DILUTION	DIED IN FIVE MINUTES. PARALYZED AND DIED WITHOUT FIRST AND SECOND STAGES	
1 : 25	+++	
1 : 50	+	
1 : 100	0	

AGGLUTININ-DILUTION	DIED IN SIX MINUTES	CONTROL, SIX MINUTES
1 : 25	++++	++++
1 : 50	+++	+++
1 : 100	++	++
1 : 200	0	+
1 : 225		?
1 : 330		0

AGGLUTININ-DILUTION	TYPICAL ANAPHYLAXIS. SAMPLE, ONE HOUR	
1 : 25	++	
1 : 50	+	
1 : 100		

AGGLUTININ-DILUTION	SEVERE SHOCK. SAMPLE, TWO HOURS	CONTROL, TWO HOURS
1 : 25	0	0
1 : 50	0	0

AGGLUTININ-DILUTION	VERY SLIGHT SHOCK. INJECTION MAY WELL HAVE BEEN INTO PERICARDIUM. SAMPLE TWO HOURS	CONTROL, TWO HOURS
1 : 25	+	+
1 : 50	0	0



*Intravenous injection. Animal bore and raised two young after sensitization.  
Upon injection it coughed once and shivered but anaphylaxis was doubtful*

AGGLUTININ-DILUTION	SAMPLE, FIVE HOURS	CONTROL, FIVE HOURS
1 : 25	++++	++++
1 : 50	++++	+
1 : 100	+++	0
1 : 200	++	
1 : 225	+	
1 : 330	0	

*Intracardiac injection*

AGGLUTININ-DILUTION	BARELY OUTLIVED SHOCK SAMPLE, TWENTY-FOUR HOURS	CONTROL, TWENTY-FOUR HOURS
1 : 25	0	++++
1 : 50	0	++
1 : 100		0
AGGLUTININ-DILUTION		CONTROL, TWENTY-FOUR HOURS
1 : 25		0
AGGLUTININ-DILUTION		CONTROL, TWENTY-FOUR HOURS
1 : 25		0

There were two more controls at forty-eight hours, which showed no agglutinin.

The conclusions to be drawn from these results are:

1. Individual variation in reaction is so great that experiments in this line of work require statistical control and must be carried out with large numbers of animals to give definite conclusions.

2. In general a foreign serum disappears more quickly from the blood of an animal sensitized to that serum than from that of a normal animal.

3. It disappears more quickly from the blood of highly reacting animals than from that of slightly reacting animals.

4. These experiments corroborate those of Römer and Viereck.

I wish to express my thanks to Dr. Thorvald Madsen for so kindly affording me the courtesies of his laboratory, and to Dr. Oluf Thompson for direction and assistance in the carrying out of this work.



# THE SPECIFIC AND NON-SPECIFIC ACTION OF RABBIT BLOOD SERUM IN THE COM- PLEMENT FIXATION TEST

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While conducting a long series of experiments relative to the transmission of *Bact. abortus* to rabbits through the ingestion of naturally infected milk, and determining the results therefrom by means of the complement fixation and agglutination tests on the blood sera of the rabbits, a non-specific complement binding or absorption property was demonstrated in the blood serum. In view of the fact that rabbits are employed in many experimental investigations, special attention should be directed toward the probable non-specific reactions in the complement fixation test.

A complete historical review in non-specific complement fixation may be found in a recent article by Kolmer and Trist (1). These investigators have demonstrated non-specific complement binding substances present in the blood sera of normal rabbits against bacterial antigens such as staphylococci, colon and typhoid bacilli. They further demonstrated conclusively that lipoids are concerned in the non-specific complement fixation reaction with normal rabbit serum.

## TECHNIQUE EMPLOYED

The complement fixation and agglutination tests are recognized as being very valuable in experimental studies of infectious abortion. The tests have been fully discussed by Hadley and Beach (2), Surface (3) and many others.

*Preparation of antigen.* The antigen used in the complement

fixation test was prepared by growing a culture of *Bact. abortus* upon plain agar for forty-eight hours at 37°C. The growth was then washed off with a solution containing phenol (0.5 per cent) and sodium chlorid (0.9 per cent) and filtered through cotton. The suspension was then standardized so that the turbidity corresponded to tube 5 of McFarland's nephelometer (4). The amount of antigen used in the final test was less than the anti-complementary unit. The antigen will retain the same titer for many months if kept at ice-box temperature.

*Serum to be tested.* The blood was collected from the rabbits by inserting a small hypodermic needle into the heart. After allowing sufficient time to elapse for separation from clot, the serum was pipetted off and inactivated by heating for one-half hour, in a water bath at 56°C. The serum was titrated twenty-four hours after being drawn.

*Hemolytic system.* The sheep-rabbit hemolytic system was used throughout. Before each series of tests the hemolytic amboceptor was titrated with a constant dose of complement against 0.5 cc. of a 2.5 per cent suspension of washed sheep erythrocytes, 1.5 cc. of physiological salt solution being added to the tubes for dilution. The most important point to be mentioned in the preparation of the hemolytic system is the manner of obtaining the complement serum. Instead of bleeding the guinea-pig to death for complement serum the animal was firmly fastened on its back to an animal board and *paracentesis cordii* was performed by inserting a small hypodermic needle between the fifth and sixth rib of the left thorax. Aspiration is unnecessary as the blood will flow freely and quickly. Six cubic centimeters of blood may be drawn from a large guinea-pig weekly without serious discomfort or injury to the animal. The serum when used in the final test was diluted 1 : 3 with physiological salt solution and employed in constant doses of 2 units.

The final test involved the use of four dilutions: 0.1, 0.04, 0.02 and 0.005 cc. of the serum to be tested, 2 units of complement, 2 units of antigen and 1.5 cc. of physiological salt solution. Two control tubes were used, one as a control against the complement absorption action of the test serum and one against

the natural hemolytic properties of the test serum. The series of tubes was then incubated for one-half hour at 37°C. in a water bath after which 2 units of hemolysin and 0.5 cc. of a 2.5 per cent suspension of sheep erythrocytes were added to the tubes. After a second incubation of one-half hour at 37°C. in the water bath the readings were made.

*The agglutination test.* The antigen for the agglutination test was prepared in the same manner as in the complement fixation test. The suspension was prepared so that the turbidity corresponded with tube 1.5 of McFarland's nephelometer. Four cubic centimeters of the bacterial suspension was placed in each of the small test tubes used and the following quantities of blood serum added: 0.1, 0.05, 0.025, 0.01 and 0.005 cc. In this way there were obtained approximate dilutions of 1:50, 1:100, 1:200 1:500 and 1:1000.

#### RESULTS

Cooledge (5) has shown that milk which reacts positively to the agglutination test although coming from an apparently normal cow contains *Bact. abortus*.

The purpose of this investigation was to determine whether rabbits would become infected and show *Bact. abortus* antibodies in their blood as a result of ingesting milk reacting positively to the agglutination and complement fixation tests. The milk was fed daily to fourteen normal female rabbits including four controls for a period of 124 days. Table 1, shows the results of the tests made at intervals of fourteen days. The reactions and partial reactions obtained by the complement fixation test were thought to be due to a passive immunity.

In order to clear up this point, four of the rabbits were fed no milk for a period of fourteen days, the serum tests being continued as before. One may readily see from table 2, that there is no marked decrease in the serum reaction after the feeding of milk was discontinued. The agglutination tests were always negative except with the serum of rabbit 7, which developed agglutinins on the one hundred and twenty-fourth day and remained positive after the feeding of milk was discontinued.

TABLE 1  
*The result of feeding naturally infected milk to rabbits*

BLOOD SERUM	REACTION OF																					
	Rabbit 1						Rabbit 2						Rabbit 3									
	Agglutinating			Complement fixation			Agglutinating			Complement fixation			Agglutinating			Complement fixation						
	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.04	0.005	0.1	0.04	0.02	0.005	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005
Before feeding.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 14 days.....	-	-	-	-	-	P	-	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-
After feeding 28 days.....	-	-	-	-	-	P	-	-	-	-	P	-	-	-	-	-	-	-	P	+	-	-
After feeding 42 days.....	-	-	-	-	-	P	-	-	-	-	P	-	-	-	-	-	-	-	P	-	-	-
After feeding 56 days.....	-	-	-	-	-	P	-	-	-	-	P	-	-	-	-	-	-	-	P	-	-	-
After feeding 70 days.....	-	-	-	-	-	P	-	-	-	-	P	-	-	-	-	-	-	-	P	-	-	-
After feeding 91 days.....	-	-	-	-	-	+	P	-	-	-	P	-	-	-	-	-	-	-	P	-	-	-
After feeding 103 days.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 124 days.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

BLOOD SERUM	REACTION OF																	
	Rabbit 4				Rabbit 5				Rabbit 6									
	Agglutinating		Complement fixation		Agglutinating		Complement fixation		Agglutinating		Complement fixation							
	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005
Before feeding.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 14 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 28 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 42 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 56 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 70 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 91 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 103 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 124 days .....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

BLOOD SERUM	REACTION OF									
	Rabbit 7					Rabbit 8				
	Agglutinating			Complement fixation		Agglutinating			Complement fixation	
	0.1	0.25	0.05	0.01	0.005	0.1	0.25	0.05	0.01	0.005
Before feeding.....	-	-	-	-	-	-	-	-	-	-
After feeding 14 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 28 days.....	-	-	-	-	-	+	-	-	-	-
After feeding 42 days.....	-	-	-	-	-	+	-	-	-	-
After feeding 56 days.....	-	-	-	-	-	+	-	-	-	-
After feeding 70 days.....	-	-	-	-	-	+	-	-	-	-
After feeding 91 days.....	-	-	-	-	-	+	-	-	-	-
After feeding 103 days.....	-	-	-	-	-	+	-	-	-	-
After feeding 124 days.....	+	+	+	P	-	+	+	+	-	-

BLOOD SERUM	REACTION OF									
	Rabbit 10					Rabbit 11				
	Agglutinating			Complement fixation		Agglutinating			Complement fixation	
	0.1	0.25	0.05	0.01	0.005	0.1	0.25	0.05	0.01	0.005
Before feeding.....	-	-	-	-	-	-	-	-	-	-
After feeding 14 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 28 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 42 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 56 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 70 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 91 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 103 days.....	-	-	-	-	-	-	-	-	-	-
After feeding 124 days.....	-	-	-	-	-	-	-	-	-	-

TABLE 1—Continued

[illegible]

BLOOD SERUM	REACTION OF														
	Control Rabbit 2 <sup>a</sup>				Control Rabbit 3 <sup>b</sup>				Control Rabbit 4 <sup>c</sup>						
	Agglutinating		Complement fixation		Agglutinating		Complement fixation		Agglutinating		Complement fixation				
	0.1	0.25	0.05		0.01	0.005	0.1		0.04	0.02	0.005	0.1	0.05	0.01	0.005
	0.1	0.25	0.05		0.01	0.005	0.1		0.04	0.02	0.005	0.1	0.25	0.05	0.01
Before feeding.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 14 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 28 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 42 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 56 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 70 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 91 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 103 days.....	L ost	—	—	—	—	—	—	—	—	—	—	—	—	—	—
After feeding 124 days.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

<sup>1</sup> Fed non-infected milk.<sup>2</sup> Fed non-infected milk.

• Fed no milk.



TABLE 2

The result of discontinuing the feeding of milk to four of the rabbits used in Table 1

MILK DISCONTINUED	RABBIT 5										RABBIT 6									
	Agglutination					Complement Fixation					Agglutination					Complement Fixation				
	.1	.25	.05	.01	.005	.1	.04	.02	.005		.1	.25	.05	.01	.005	.1	.04	.02	.005	
14 days.....	—	—	—	—	—	+	—	—	—		—	—	—	—	—	P	—	—	—	—
28 days.....	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—
42 days.....	—	—	—	—	—	P	—	—	—		—	—	—	—	—	P	—	—	—	—
56 days.....	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—
70 days.....	—	—	—	—	—	—	—	—	—		—	—	—	—	—	—	—	—	—	—
84 days.....	—	—	—	—	—	P	—	—	—		—	—	—	—	—	+	—	—	—	—

MILK DISCONTINUED	RABBIT 7										RABBIT 8									
	Agglutination					Complement Fixation					Agglutination					Complement Fixation				
	.1	.25	.05	.01	.005	.1	.04	.02	.005		.1	.25	.05	.01	.005	.1	.04	.02	.005	
14 days.....	+	+	+	—	—	+	+	+	—		—	—	—	—	—	P	—	—	—	—
28 days.....	+	+	+	—	—	+	+	P	—		—	—	—	—	—	P	—	—	—	—
42 days.....	+	+	+	—	—	+	+	P	—		—	—	—	—	—	P	—	—	—	—
56 days.....	+	+	P	—	—	+	+	+	—		—	—	—	—	—	—	—	—	—	—
70 days.....	+	+	+	P	—	+	+	+	+		—	—	—	—	—	P	—	—	—	—
84 days.....	+	+	+	—	—	+	+	+	P		—	—	—	—	—	P	—	—	—	—

The blood serum of the controls (rabbits fed upon non-infected milk and no milk) show reactions and partial reactions similar to those observed with the blood of the rabbits fed naturally infected milk.

Table 3 shows the result of feeding non-infected milk plus 5 cc. of a forty-eight hour bouillon culture of *Bact. abortus* daily to six normal female rabbits. The blood serum of each rabbit developed complement fixing and agglutinating antibodies shortly after the feeding of the mixture was begun. The antibody index was not constant. It gradually developed to a maximum, remained for a short time and gradually decreased while the mixture was being fed.

Each of the rabbits was autopsied at the termination of the experiment but no anatomical changes were found.

TABLE 3

*The result of feeding non-infected milk plus a culture of Bact. Abortus to rabbits*

BLOOD SERUM	REACTION OF																	
	Rabbit 15				Rabbit 16				Rabbit 17									
	Agglutinating		Complement fixation		Agglutinating		Complement fixation		Agglutinating		Complement fixation							
	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005
Before feeding.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 14 days.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
After feeding 28 days.....	-	-	-	-	-	P	-	-	-	-	+	P	-	-	+	+	+	-
After feeding 42 days.....	-	-	-	-	-	P	-	-	-	-	+	-	-	-	+	P	-	-
After feeding 56 days.....	-	-	-	-	-	+	P	-	-	-	-	-	-	-	+	-	-	-
After feeding 70 days.....	+	-	-	-	-	+	+	-	-	-	P	-	-	-	-	-	-	-
After feeding 84 days.....	-	-	-	-	-	+	P	-	-	-	-	-	-	-	+	+	-	-

BLOOD SERUM	REACTION OF																	
	Rabbit 18				Rabbit 19				Rabbit 20									
	Agglutinating		Complement fixation		Agglutinating		Complement fixation		Agglutinating		Complement fixation							
	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005	0.1	0.25	0.05	0.01	0.005	0.1	0.04	0.02	0.005
Before feeding.....	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
After feeding 14 days.....	+	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	+	-
After feeding 28 days.....	+	+	+	-	-	+	+	P	-	-	+	+	+	-	+	+	+	-
After feeding 42 days.....	+	+	P	-	-	+	+	+	-	-	+	+	P	-	+	+	+	-
After feeding 56 days.....	+	+	+	-	-	+	+	+	-	-	+	+	+	-	+	+	+	-
After feeding 70 days.....	P	-	-	-	-	-	P	-	-	-	+	+	+	-	+	+	+	-
After feeding 84 days.....	-	-	-	-	-	-	-	-	-	-	+	P	-	-	+	+	+	-

## DISCUSSION

If the complement-binding property possessed by the sera of the rabbits shown in table 1 is due to specific antibodies, the total amount of antibodies present in a given serum when mixed with a sufficient amount of antigen and incubated at 37°C. should be absorbed or fixed by the antigen. The serum when again titrated should be void of any complement fixing property whatever. The author selected several of the rabbit sera tested in table 1, together with two positive bovine sera in order to confirm or disprove the above statement.

Two cubic centimeters of each serum were mixed with a dense suspension of *Bact. abortus* antigen and incubated for three hours at 37°C. The mixture was then centrifuged and the supernatant serum drawn off and titrated. The rabbit sera in the presence of antigen absorbed the complement in the same dilutions as before. The two bovine sera showed no complement-binding properties whatever; complete hemolysis took place in each dilution. The serum control tubes containing the maximum amount of serum plus complement showed complete hemolysis. The fact that old serum or a serum in which hemolysis has occurred will inhibit hemolysis is well known. This fact, however, must be excluded as a factor in these tests as the serum void of hemolysis was tested twenty-four hours after being drawn.

A non-specific complement binding substance is usually produced when rabbit's blood serum and a bacterial antigen of *Bact. abortus* are mixed and incubated together.

The author, at present, can offer no explanation of this non-specific peculiarity of rabbit blood serum.

## SUMMARY

There is not sufficient evidence to condemn naturally infected milk as dangerous to rabbits by ingestion since it has shown no pathogenic action and no antigenic action.

A non-specific absorption of complement takes place in the presence of inactivated rabbit blood serum and a bacterial antigen of *Bact. abortus*.

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# ANALYSIS OF THE ANAPHYLACTIC AND IMMUNE REACTIONS BY MEANS OF THE ISOLATED GUINEA-PIG LUNGS<sup>1</sup>

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Analyses of the anaphylactic reaction by means of kymograph experiments with the isolated anaphylactic uterus are of uncertain interpretation, due to the fact that in such tests the foreign protein and reacting sera are introduced directly into the extra-vascular tissue spaces. In the anaphylactic reaction as it takes place in the animal body, the foreign protein is brought to the reacting tissues by the blood stream. The vascular endothelium is the fixed tissue primarily affected, and it is only after a breaking down of the endothelial defense, or after a possible functional coöperation of the endothelial cells, that the foreign protein or its products, is brought into contact with the extra-vascular tissues.

Two years ago, we tried to meet this objection by studying the anaphylactic uterus by perfusion methods (1). On account of the experimental difficulties in such perfusions, however, the attempts were discontinued.

Last year, we applied perfusion methods to the isolated anaphylactic heart (2). The rabbit heart can be readily studied by such methods. These analyses showed that the anaphylactic reaction in the rabbit is a purely humoral reaction, due solely to toxic substances formed or liberated by an interaction of the foreign protein and the anaphylactic blood. The myocardium

<sup>1</sup> Presented before the Society of American Bacteriologists, New Haven, Conn., December 27, 1916, and before the American Society for Experimental Pathology, New York City, December 28, 1916.

of the anaphylactic rabbit showed no hypersensitiveness when compared, under identical conditions, with the normal rabbit myocardium.

These results are not in accord with the conclusions of Weil and others drawn from a study of the isolated guinea-pig uterus. This year, we have, therefore, renewed perfusion experiments with guinea-pig tissues. The work here reported consists of certain introductory results drawn from a study of the isolated guinea-pig lungs.

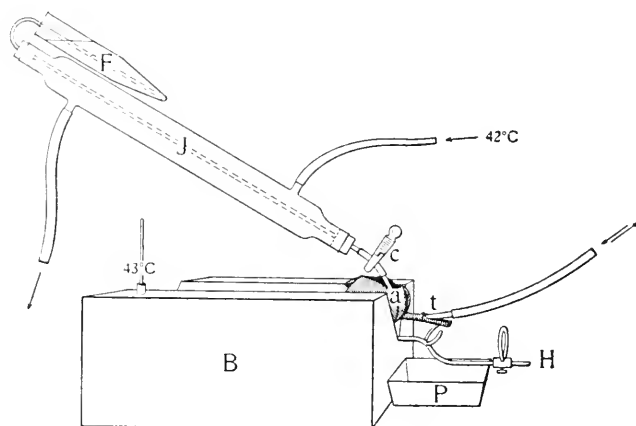


FIG. 1. PERFUSION APPARATUS

*F*, Perfusion fluid; *J*, warming jacket; *a*, cannula in pulmonary artery. Perfusion fluid escapes from the open left auricle into the pan, *P*. Lungs alternately inflated and allowed to collapse by air forced in through the tracheal cannula, *t*.

*Method.* The apparatus used in the pulmonary perfusions is shown semi-diagrammatically in figure 1. The guinea-pig (not shown in the sketch) is tied to a special copper box (*B*), containing water heated to 43°C., and provided with a head-holder (*H*) and leg-holders (not shown in the sketch). The animal is partially exsanguinated by bleeding from the carotid, the ductus arteriosus is ligated, and a citrated cannula tied into the pulmonary artery (*a*). Through this cannula the perfusion fluid is passed into the lungs, under controllable temperature and pressure. After passing through the lungs, the fluid is allowed to escape from the open left auricle. In this way the lungs

can be washed free from blood, and tested with various serum and blood mixtures.

During the perfusion, the lungs are alternately expanded and allowed to contract by air forced into the tracheal cannula (*t*), in a manner simulating the normal respiratory movements. Note is made of the resulting changes in resistance to inflation and in the promptness and completeness of the expiratory collapse.

*Material.* The animals studied by this method were:

(a) *Anaphylactic guinea-pigs.* 200 gram guinea-pigs, sensitized by a single subcutaneous injection with 0.01 to 0.05 cc. goat serum; tested fourteen to sixty days after the injection.

(b) *Immune guinea-pigs.* 200 gram guinea-pigs, given three to five subcutaneous injections, at four to five day intervals, of 0.05 to 0.1 cc. goat serum; tested eight to twenty days after the final injection.

*Record.* In recording these observations, the following symbols are used:

(a) *Lung.*

N = Normal lungs

A = Anaphylactic lungs ( $A_{14}$  = 14-day anaphylactic lungs,  $A_{28}$  = 28-day anaphylactic lungs, etc.)

I = Immune lungs ( $I_3$  = 3 injections with goat serum,  $I_5$  = 5 injections, etc.)

(b) *Perfusion fluid.*

Lck = Locke's solution

NB = Defibrinated normal guinea pig blood (50 per cent NB = 50 per cent blood in Locke's solution, etc.)

NS = Normal guinea pig serum (33 per cent NS = 33 per cent serum in Locke's solution, etc.)

AB, AS = Anaphylactic blood, anaphylactic serum

IB, IS = Immune blood, immune serum

GS = Goat serum

In the tests here reported, the mixtures of blood, goat serum, etc., were made immediately before the perfusions, since we were concerned in this paper only with the immediate pulmonary reactions, and not with the possible production of toxic substances by a prolonged incubation of such mixtures.

(c) *Alveolar fixation:*

0 = No change in pulmonary elasticity; lungs readily inflated, fully collapsible on expiration.

- 1 = Beginning fixation; lungs show a slight resistance to inflation; remain slightly expanded on expiration.
- 2 = Distinct fixation; lungs show a distinct resistance to inflation, remain half expanded on expiration.
- 3 = Marked fixation; lungs inflated with difficulty, remain three-quarters expanded on expiration.
- 4 = Total fixation; lungs of liver-like firmness, can neither be expanded nor collapsed by changes in air pressure in the tracheal cannula.

(d) *Vascular resistance* (vasc.):

- 0 = No diminution in rate of flow of perfusion fluid.  
 $\pm$  = Diminished rate of flow  
 + = Cessation of flow.

(e) *Pulmonary edema* (edem.):

- 0 = No edema of lungs  
 $\pm$  = Slight edema  
 + = Marked edema, frothy fluid escaping from trachea.

#### ANAPHYLACTIC REACTION

*Normal lungs.* If the lungs of a normal guinea-pig are perfused with 50 per cent defibrinated normal blood containing 2 to 4 per cent goat serum, no change in the resistance to inflation, nor in the promptness or completeness of the expiratory collapse are observed (N, table 1). There is no increased resistance to perfusion and no edema.

*Anaphylactic lungs.* If the lungs of an anaphylactic guinea-pig are similarly perfused with 50 per cent defibrinated anaphylactic blood containing 2 to 4 per cent goat serum, the lungs are thrown into a prompt anaphylactic reaction (A, table 1).

TABLE 1  
*Comparison of normal and anaphylactic lungs*

LUNGS	PERFUSION FLUID	ALVEOLAR FIXATION										VASC.	EDEM.
		1 min.		2 min.		3 min.		4 min.		5 min.			
N	50% NB + 2% GS....	0	0	0	0	0	0	0	0	0	0	0	
A	50% AB + 2% GS....	0	1	3	4	4	4	4	4	4	4	$\pm$ 0	



Within forty-five seconds, a slight increase in the resistance to inflation is usually observed, together with a decrease in the completeness of the expiratory collapse. Within seventy-five seconds the lungs usually lose three-quarters of their original expiratory collapse, and usually becoming completely immobilized within ninety seconds. The lungs can now neither be further dilated nor collapsed by changes in air pressure in the tracheal cannula.

In exceptionally strong reactions, there is now observed a decrease in the rate of the flow of the perfusion fluid; in very rare cases, an edema.

## REACTION ANALYSED

(A) *Fourteen-day anaphylactic guinea-pig*

(1) *Cellular reaction.* If the lungs of a 14-day anaphylactic guinea-pig are washed free from anaphylactic blood by a preliminary perfusion with Locke's solution, and are then perfused with:

(a) Locke's solution containing 2 per cent goat serum (Lck + 2 per cent GS), (b) 50 per cent normal blood containing 2 per cent goat serum (50 per cent NB + 2 per cent GS), or (c) 33 per cent normal serum containing 2 per cent goat serum (33 per cent NS + 2 per cent GS), the lungs are invariably thrown into a typical anaphylactic reaction (1, 2, 3, table 2). The reaction

TABLE 2  
*Reactions of 14-day anaphylactic guinea-pigs*

LUNGS	PERFUSION FLUID	ALVEOLAR FIXATION										VASC.	EDEM.
		1 min.		2 min.		3 min.		4 min.		5 min.			
1. A <sub>14</sub>	Lck + 2% GS.....	0	0	0	1	2	3	3	4	4	4	0	0
2. A <sub>14</sub>	50% NB + 2% GS..	0	0	1	2	3	4	4	4	4	4	0	0
3. A <sub>14</sub>	33% NS + 2% GS..	0	0	0	1	3	4	4	4	4	4	0	0
4. N	50% AB + 2% GS..	0	0	0	0	1	2	3	3	3	3	0	≠
5. N	33% AS + 2% GS..	0	0	0	0	0	1	1	2	2	3	0	0
6. A <sub>14</sub>	50% AB + 2% GS..	0	1	3	4	4	4	4	4	4	4	≠	0
7. A <sub>14</sub>	33% AS + 2% GS..	0	0	2	3	3	4	4	4	4	4	0	0

usually begins within ninety seconds and usually leads to a complete alveolar fixation within three minutes.

Control perfusions (not shown in table) of normal lungs with the same serum mixtures, give no reactions.

(2) *Humoral reaction.* If the lungs of a normal guinea-pig are perfused with:

(a) 50 per cent 14-day anaphylactic blood containing 2 per cent goat serum (50 per cent AB + 2 per cent GS), or (b) 33 per cent 14-day anaphylactic serum containing 2 per cent goat serum (33 per cent AS + 2 per cent SG), the lungs are invariably thrown into a typical anaphylactic reaction (4, 5, table 2). The reaction usually begins within two minutes, usually reaches a maximum within four minutes, but rarely leads to a complete alveolar fixation.

Control perfusions (not shown in table) of normal lungs with normal blood and normal serum mixtures, give no reactions.

(3) *Combined reaction.* If the lungs of a 14-day anaphylactic guinea pig are perfused with:

(a) 50 per cent 14-day anaphylactic blood containing 2 per cent goat serum (50 per cent AB + 2 per cent GS), or (b) 33 per cent 14-day anaphylactic serum containing 2 per cent goat serum (33 per cent AS + 2 per cent GS), the lungs are invariably thrown into a typical anaphylactic reaction (6, 7 table 2). The reaction is always prompt and stronger than either the cellular or the humoral reaction above recorded, and usually leads to a complete alveolar fixation within ninety seconds.

*Conclusion.* The fatal bronchial spasm in the 14-day anaphylactic guinea-pig is due, partly to cellular hypersensitiveness, partly to humoral anaphylaxis.

#### (B) *Four-week anaphylactic guinea-pig*

Similar tests with 4-week anaphylactic guinea pigs, are recorded in table 3.

The table shows that the cellular hypersensitiveness of the 4-week anaphylactic guinea-pig is usually greater than that of the 14-day anaphylactic guinea pig (1, 2, 3, table 3). The humor-

TABLE 3  
*Reactions of 4-week anaphylactic guinea-pigs*

[illegible]

al reaction, however, is always weak and usually absent (4, 5, table 3).

*Conclusion.* The fatal bronchial spasm in the 4-week anaphylactic guinea pig is usually due solely to cellular hypersensitiveness.

## IMMUNE REACTION

Similar tests with the lungs of guinea-pigs immunized with repeated doses of goat serum are recorded in table 4.

The table shows that the lungs of an immunized guinea-pig show a cellular hypersensitiveness (1, 2, 3, table 4), usually greater than the cellular hypersensitiveness of the 14-day anaphylactic guinea-pig.

TABLE 4  
*Reactions of immune guinea-pigs*

[illegible]

The element of humoral anaphylaxis, however, is always lacking in the immune guinea pig (4, 5, table 4).

Humoral anaphylaxis is not only absent in the immune guinea-pig, but its place is evidently taken by a humoral immunity, since immune blood and immune serum mixtures do not produce anaphylactic reactions in the guinea-pigs' own hypersensitive tissues (6, 7, table 4).

Further proof of this humoral immunity is furnished in table 5, where it is seen that immune blood and immune sera prevent the anaphylactic reactions in 14-day and 4-week anaphylactic lungs.

*Conclusion.* The immune guinea-pig shows a seemingly paradoxical phenomenon, a fixed cellular hypersensitiveness, inhibited or protected by a humoral immunity.

TABLE 5  
*Humoral immunity*

LUNGS	PERFUSION FLUID	ALVEOLAR FIXATION										VASC.	EDEM.
		1 min.		2 min.		3 min.		4 min.		5 min.			
1. A <sub>14</sub>	50% IB + 2% GS...	0	0	0	0	0	0	0	0	0	0	0	0
2. A <sub>28</sub>	50% IS + 2% GS....	0	0	0	0	0	0	0	0	0	0	0	0

#### SUMMARY

Analyses of the anaphylactic and immune reactions by means of perfusion experiments with the isolated guinea-pig lungs show that we are here concerned with three essential factors:

(a) *Cellular hypersensitiveness*, or the anaphylactic response of the hypersensitive fixed pulmonary tissues,

(b) *Humoral anaphylaxis*, or the chemical response (anaphylo-toxin formation) of the anaphylactic blood, and

(c) *Humoral immunity*, or the inhibiting or protecting action of the immune blood.

In the 14-day anaphylactic guinea-pig, the fatal bronchial spasm is due, in part to fixed cellular hypersensitiveness, in part to humoral anaphylaxis.

In the 4-week anaphylactic guinea-pig, the fixed cellular

hypersensitiveness is usually greater than that of the 14-day anaphylactic guinea-pig. The humoral reaction, however, is usually absent. The fatal bronchial spasm in the 4-week anaphylactic guinea-pig is usually due solely to fixed cellular hypersensitiveness.

The immune guinea-pig usually shows a fixed cellular hypersensitiveness greater than that of the 14-day anaphylactic guinea pig. A fatal bronchial spasm is prevented in the immune guinea-pig, however, by the inhibiting or protecting action of the immune blood.

The immune guinea-pig, therefore, shows a seemingly paradoxical phenomenon, the coexistence of a fixed cellular hypersensitiveness and a humoral immunity.

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# THE BACTERIOLYTIC POWER OF NORMAL AND IMMUNE RABBIT SERUM FOR TYPHOID BACILLI AND THE INFLUENCE OF THE INTRAVENOUS INJECTION OF VACCINE UPON THE SAME

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Since the demonstration by Ichikawa (1), Kraus (2) and others that typhoid fever patients are in many instances cured by crisis after an intravenous injection of killed typhoid bacilli, a number of hypotheses have been advanced to explain the striking phenomenon. Bull (3) has recently studied the influence of the injection of typhoid bacilli into normal and immune rabbits upon the content of the serum in various antibodies. He found no reduction in the concentration of the antibodies one hour, five hours, or twenty-four hours after the injection. However, he published only two experiments dealing with the bacteriolytic antibodies; each of the rabbits received  $\frac{1}{2}$  of a slant of typhoid bacilli intravenously and in one instance the serum of the rabbit killed 500 typhoid bacilli per cubic centimeter before the injection, 50,000 per cubic centimeter five hours after the injection and 5,000,000 per cubic centimeter twenty-four hours after the injection; in the other instance, the serum killed 50,000 typhoid bacilli per cubic centimeter before the injection, 250,000 per cubic centimeter three hours after the injection and 5,000,000 per cubic centimeter five hours after the injection. He says: "It is convenient to imagine that the beneficial effects said to follow the treatment of typhoid fever by means of the intravenous injection of specially prepared typhoid bacilli may be ascribed to this rapid mobilization of antibodies." When Bull's article appeared we were occupied

with experiments designed to throw light upon the mechanism of the cure of typhoid fever by the intravenous injection of vaccine and some of these experiments were similar in character to his. In view of his striking results it seemed advisable to investigate more extensively and by more accurate methods the influence of intravenous injections of vaccine upon the bactericidal power of rabbit serum.

Buxton (4) made a very careful study of the bacteriolytic action of normal and immune rabbit serum and showed that, while normal rabbit sera varied greatly in their bactericidal power, 1 cc. might be expected to kill about 1,000,000 typhoid bacilli or about 50,000,000 paratyphoid bacilli. He immunized rabbits by injecting them with typhoid bacilli every ninth day and tested the bacteriolytic power of their sera on the seventh day after each inoculation; the undiluted serum did not kill typhoid bacilli after the rabbit had received two or more injections. In other words the undiluted serum of the slightly immunized rabbit killed typhoid bacilli, while that of the more highly immunized rabbit did not. It seemed, therefore, of interest to determine the influence of intravenous injections of killed typhoid bacilli upon the bactericidal power of the sera of both slightly and highly immunized rabbits.

Our technique in determining the bacteriolytic power of sera was modelled very closely after that of Buxton. The blood was drawn from the ear-vein of the rabbit, allowed to stand about three hours at room temperature and placed in the ice box. On the following morning the serum was pipetted off and centrifugated to remove the blood cells. It was then distributed in 1 cc. amounts in test tubes 1.5 cm. in diameter and 13 cm. high. Typhoid bacilli from a twenty-four hour old agar culture were suspended in saline solution and compared as to cloudiness with a formalinized stock suspension containing 500,000,000 bacilli to the cubic centimeter. Half cubic centimeter amounts, of appropriate dilutions of the suspensions were plated in agar to determine more accurately the dosage of bacilli used. Dilutions of the suspension were prepared in broth and three drops of those dilutions that would yield the desired doses of



typhoid bacilli were inoculated into each of the tubes of the serum, care being taken that the drops fell into the serum and not upon the wall of the test tube. The number of drops to the cubic centimeter delivered by the pipette was counted; three drops usually amounted to 0.1 cc. The inoculated serum tubes were then gently shaken. One loop from the tubes containing suitable doses was inoculated over the surface of an agar plate. The serum tubes were incubated for five hours, after which they were gently shaken and one loop from each tube was inoculated upon the surface of an agar plate; the incubation was extended to a period of eighteen to twenty-four hours and one loop was again inoculated upon the surface of a quadrant of an agar plate. The plates were opened and left face down in the incubator for one-half hour before inoculation in order to dry the surface of the agar. The number of colonies developing upon the plates was recorded after twenty-four hours' incubation.

The results of our tests with normal rabbit serum are given in table 1. Sixteen, or nearly one-half of the sera examined, killed a million or more typhoid bacilli per cubic centimeter; six sera killed between 100,000 and 1,000,000; two yielded doubtful results because sufficiently small doses were not inoculated; and three killed less than 10,000 per cubic centimeter. These experiments extended over a period of about six months, and for the majority of the tests a typhoid culture isolated from the blood of a patient about four months previously was used; for the later experiments a freshly isolated culture was employed.

Buxton found that there was no appreciable difference in the susceptibility of freshly isolated and old typhoid cultures. We have observed considerable differences in susceptibility in different strains which did not depend, however, on the length of the time since isolation upon culture media.

We studied the influence of the intravenous injection of typhoid bacilli in normal rabbits upon the bacteriolytic power of the serum. Rabbit 59 received living typhoid bacilli, the others bacilli heated one-half hour at 60°C. The rabbit was first bled from the ear-vein, then injected intravenously with the

TABLE 1

*Showing the bactericidal power of normal rabbit sera for typhoid bacilli*

RABBIT NO.	DOSAGE OF BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF BACILLI SURVIVING IN ONE LOOP OF SERUM		APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
		After 5 hours' incubation	After 24 hours' incubation	
23	50,000,000	$\alpha$	$\alpha$	10,000,000
	25,000,000	f.num.	$\alpha$	
	10,000,000	200	3	
	1,000,000	4	0	
	100,000	0	0	
58	8,000,000	20		8,000,000
	800,000	2		
	80,000	0		
31	10,000,000	36	f. num.	10,000,000
	1,000,000	4	22	
	100,000	0	0	
62	40,000,000	num.	$\alpha$	8,000,000
	8,000,000	23	0	
	1,500,000	9	0	
	320,000	0	0	
20+	20,000,000	78	num.	8,000,000
	8,000,000	49	f. num.	
	800,000	2	0	
65	26,000,000	134	$\alpha$	6,000,000
	13,000,000	17	$\alpha$	
	6,000,000	5	num.	
	3,000,000	3	9	
	1,500,000	0	7	
	750,000	0	0	
68	2,300,000	127	41	2,300,000
	1,000,000	28	24	
	460,000	16	66	
	230,000	3	0	
33	30,000,000	num.	$\alpha$	2,500,000
	12,000,000	74	$\alpha$	
	6,000,000	65	num.	
	2,500,000	21	f. num.	
	600,000	11	0	

TABLE 1—*Continued*

RABBIT NO.	DOSAGE OF BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF BACILLI SURVIVING IN ONE LOOP OF SERUM		APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
		After 5 hours' incubation	After 24 hours' incubation	
25	50,000,000	α	α	1,000,000+
	25,000,000	f. num.	α	
	10,000,000	200	f. num.	
	1,000,000	11	0	
	100,000	1	0	
24	50,000,000	α	α	1,000,000+
	25,000,000	f. num.	α	
	10,000,000	200	f. num.	
	1,000,000	17	1	
	100,000	1	0	
28	10,000,000	69	α	1,000,000
	1,000,000	7	150	
	100,000	0	0	
22	50,000,000	α	α	1,000,000
	25,000,000	f. num.	α	
	10,000,000	250	α	
	1,000,000	11	f. num.	
	100,000	4	0	
14	1,000,000	10	f. num.	1,000,000
	100,000	2	0	
13	50,000,000	num.	α	1,000,000
	25,000,000	311	α	
	10,000,000	217	num.	
	5,000,000	59	f. num.	
	1,000,000	9	9	
	100,000	1	0	
95	1,000,000	3	2	1,000,000
13	10,000,000	f. num.	num.	1,000,000
	1,000,000	8	f. num.	
	100,000	0	0	
26	10,000,000	f. num.	α	1,000,000—
	1,000,000	24	α	
	100,000	1	0	

TABLE 1—*Continued*

RABBIT NO.	DOSAGE OF BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF BACILLI SURVIVING IN ONE LOOP OF SERUM		APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
		After 5 hours' incubation	After 24 hours' incubation	
39	30,000,000	num.	α	600,000
	3,000,000	63	α	
	600,000	22	f. num.	
	300,000	19	3	
	30,000	3	0	
30	500,000	22	15	500,000
	50,000	1	0	
25	14,000,000	α	α	140,000+
	1,400,000	num.	f. num.	
	140,000	0	0	
16+	10,000,000	171	α	100,000+
	100,000	0	0	
16	100,000	0	26	100,000
	1,000	0	0	
20	1,000,000		α	100,000—
	100,000		f. num.	
	1,000		0	
81	26,000,000	α	α	100,000—
	4,000,000	f. num.	α	
	600,000	250	α	
	100,000	58	α	
	16,000	0	0	
15	30,000,000	num.	α	60,000
	6,000,000	207	f. num.	
	600,000	154	f. num.	
	60,000	38	31	
	600	0	0	
83	900,000	α		72,000
	360,000	num.		
	180,000	num.		
	72,000	85		
32	6,000,000	num.	α	60,000—
	2,500,000	num.	α	
	600,000	f. num.	num.	
	60,000	55	f. num.	

TABLE 1—*Concluded*

RABBIT NO.	DOSAGE OF BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF BACILLI SURVIVING IN ONE LOOP OF SERUM		APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
		After 5 hours incubation	After 24 hours incubation	
59	550,000	α	α	5,500—
	55,000	f. num.	α	
	5,500	89	f. num.	
80	110,000	num.	α	3,000—
	18,000	66	α	
	3,000	34	α	
18	150,000	num.	α	1,500
	15,000	120	f. num.	
	1,500	7	17	
	150	3	0	
64	6,000,000	num.	α	?
	3,000,000	297	num.	
20	1,700,000	num.	α	?
	170,000	f. num.	α	
29	500,000	300	α	50,000—
	50,000	32	f. num.	
	5,000	3	69	

typhoid bacilli, and finally, after the interval specified in the table, was bled a second time. The blood was allowed to clot at room temperature and was then placed in the ice box. The sera drawn before and after the injection of the bacilli were tested simultaneously against the same suspension of typhoid bacilli. The doses of typhoid bacilli injected varied between 15,000,000 and 1,000,000,000 and, as is seen from table 2, caused no appreciable change in the bacteriolytic property of the normal serum.

The figures in parentheses indicate for each dose of typhoid per cubic centimeter of serum the number of bacilli surviving in one loop of the serum after twenty-four hours incubation.

It is seen from table 3 that after the rabbits had received three or more injections their sera did not inhibit the smallest dose of bacilli used in the tests. Rabbit 75 after five inoculations

is not an exception, since its immunity was allowed to recede during the four months' interval that elapsed before the test. This confirms the observation of Buxton and others that the determination of the bacteriolytic titre of a serum by measur-

TABLE 2

*Showing the influence of the intravenous injection of typhoid bacilli upon the bactericidal property of normal rabbit serum for typhoid bacilli*

RABBIT NO.	NUMBER OF BACILLI INJECTED	RABBIT BLED AFTER	NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	
			Before the injection	After the injection
		<i>hrs.</i>		
58	400 million	24	8,000,000..... (20)	8,000,000..... (22)
			800,000..... (2)	800,000..... (4)
			80,000..... (0)	80,000..... (0)
59	300 million (living)	24	55,000..... (α)	55,000..... (numerous)
			5,500 (fairly numerous)	5,500. (fairly numerous)
83	1,000 million	3	180,000..... (650)	180,000..... (650)
			72,000..... (85)	72,000..... (252)
62	750 million	5	40,000,000..... (α)	40,000,000..... (α)
			8,000,000..... (0)	8,000,000..... (56)
				1,600,000..... (0)
68	15 million	3	2,300,000..... (41)	2,300,000..... (0)
			230,000..... (0)	
64	15 million	3	3,000,000..... (444)	3,000,000..... (numerous)
65	17 million	3	13,000,000..... (α)	13,000,000..... (α)
			6,000,000..... (numerous)	6,000,000..... (α)
			3,000,000..... (9)	3,000,000..... (180)
			750,000..... (0)	750,000..... (0)
		29		6,000,000..... (α)
				2,000,000..... (numerous)
				650,000..... (47)

ing the greatest dilution at which it will kill bacilli after the addition of complement does not indicate at all the bacteriolytic power of the blood plasma in the living animal.

The influence of the injection of large doses of typhoid bacilli intravenously in highly immunized rabbits upon the lytic action of the serum appears from table 3 to be nil; the serum does not inhibit the smallest doses of typhoid bacilli employed, either before or after the injection of the bacilli. If we include rabbit 75 in the group of slightly immune rabbits (i.e., those having received only one or two injections) then in six of the fourteen tests there is a distinct, though not very great, increase in the bacteriolytic action of the serum following the intravenous injection of killed typhoid bacilli and in only one instance, rabbit 65 did the serum kill better before than after the injection; in the other seven instances no change was effected by the injection of the bacilli.

According to Buxton normal rabbit serum has little or no effect on *B. coli*. Table 1 shows that our strain of *B. coli* communis, which had been carried for several years on nutrient agar is killed more readily than *B. typhosus*. This table shows also that the sera of slightly immune rabbits kills *B. coli* some what more readily several hours after the intravenous injection of killed *B. coli* than they did previous to the injection, the rabbits immune to *B. coli* acting in this respect like the slightly immune typhoid rabbits

It is seen from table 5 that paratyphoid bacilli vary greatly in their susceptibility to normal rabbit serum, paratyphoid B being less readily killed than paratyphoid A. Buxton found that 1 cc. of the serum would kill about 40,000,000 paratyphoid bacilli, so presumably he was working with a strain of *B. paratyphosus* A. The serum of rabbit 19, which was highly immunized against Paratyphoid A. Driv. did not kill this organism even in small doses. The strains Paratyphoid A. Driv., Paratyphoid A. Gr. and Paratyphoid B. Gach. were isolated in this laboratory from the blood of human cases a few weeks or months before their employment in these experiments; the others were old laboratory strains.

TABLE 3  
*Showing the influence of the intravenous injection of typhoid bacilli upon the bactericidal property of typhoid-immune rabbit serum for typhoid bacilli*

RABBIT NO.	NUMBER OF PREVIOUS INJECTIONS	INTERVAL SINCE LAST INJECTION	NUMBER OF BACILLI INJECTED	RABBIT BLEED AFTER	NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	
					Before the injection	After the injection
69	1	5 days	15 million	5 hours	2,300,000..... (0)	2,300,000..... (0)
64	1	10	700 million	3	2,000,000..... (α) 660,000..... (28) 220,000..... (0)	2,000,000..... (200) 660,000..... (24)
65	1	12	700 million	3	14,000,000..... (92) 5,000,000..... (24) 2,000,000..... (0)	14,000,000..... (α) 5,000,000..... (400) 2,000,000..... (0)
62	1	24	2,200 million	8	(Contaminated)	30,000,000..... (α) 5,000,000..... (38) 1,000,000..... (0)
58	1	43	100 million	5	6,000,000..... (num.) 2,400,000..... (150) 600,000..... (0)	12,000,000..... (num.) 2,400,000..... (28) 600,000..... (0)
				17		12,000,000..... (f. num.) 2,400,000..... (0)



## BACTERIOLYTIC POWER OF RABBIT SERUM

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59	1	48	?	17	600,000..... (f. num.)	2,400,000..... (num.) 600,000..... (0)
69	2	5	22 million	3	6,000,000..... (143) 3,000,000..... (29)	6,000,000..... (194) 3,000,000..... (17) 750,000..... (0)
63	2	8	700 million	29		20,000,000..... (α) 6,000,000..... (74) 600,000..... (5)
65	2	10	Large dose	5 24	1,000,000..... (α)	1,000,000..... (α) 27,000..... (α)
64	2	10	Large dose	5	64,000..... (α)	64,000..... (α)
54	2	15	2,000 million	5	64,000..... (α)	64,000..... (α)
				8	1,000,000..... (α) 100,000..... (f. num.) 10,000..... (5) 1,000..... (0)	10,000,000..... (α) 1,000,000..... (f. num.) 100,000..... (0)
52	2	29	8,500 million	24	50,000..... (α) 5,000..... (num.)	500,000..... (α) 50,000..... (57) 5,000..... (0)

TABLE 3—Continued

RABBIT NO.	NUMBER OF PREVIOUS INJECTIONS	INTERVAL SINCE LAST INJECTION	NUMBER OF BACILLI INJECTED	RABBIT BLEED AFTER	NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	
					Before the injection	After the injection
62	2	40	1,000 million	<i>hours</i> 5	2,400,000..... (α)	6,000,000..... (α)
					600,000..... (8)	2,400,000..... (f. num.)
						600,000..... (1)
80	2	22	8,500 million	24		12,000,000..... (f. num.)
						2,400,000..... (40)
						600,000..... (0)
69	3	5	15 million	24	500,000..... (α)	500,000..... (400)
					50,000..... (80)	50,000..... (0)
					Contaminated	
65	3	27			16,000..... (α)	
64	3	30			16,000..... (α)	
54	3	42	8,500 million	24	50,000..... (α)	50,000..... (α)
					5,000..... (num.)	5,000..... (f. num.)
82	3	8	5,000 million	5	10,000..... (α)	10,000..... (α)
					1,000..... (52)	1,000..... (54)
69	4	8	700 million	24		27,000..... (α)

82	4	22	500 million	24	5,000..... (α)	5,000..... (α)
75	5	120	40 million	5	400,000..... (200) 80,000..... (200)	2,000,000..... (num.) 400,000..... (14) 80,000..... (0)
75	6	15	2,500 million	5	16,000..... (α) 3,000..... (f. num.)	3,000..... (α)
76	7	31	15 million	5	80,000..... (α)	80,000..... (α)
74	6	35	100 million	5	80,000..... (α)	80,000..... (α)
74	7	30	1,200 million	5	600..... (α) 60..... (num.) 6..... (4)	600..... (α) 60..... (num.) 6..... (6)

TABLE 4

*Showing the bactericidal action of normal and B. coli-immune rabbit sera upon B. coli and the influence of intravenous injections of B. coli upon the bactericidal property of the sera*

RABBIT NO.	NUMBER OF PREVIOUS INJECTIONS	INTERVAL SINCE LAST INJECTION <sup>a</sup>	NUMBER OF B. COLI INJECTED	RABBIT BLED AFTER	NUMBER OF B. COLI KILLED PER CUBIC CENTIMETER OF SERUM	
					Before the injection	After the injection
		<i>days</i>		<i>hours</i>		
80	Normal				300,000,000..... (6) 86,000,000..... (0)	
81	Normal				160,000,000..... (0)	
60	Normal		75 million	5	386,000,000..... (∞) 64,000,000..... (1)	386,000,000..... (numerous) 64,000,000..... (1)
60	1	8	75 million	8½	180,000,000..... (∞) 30,000,000..... (100) 5,000,000..... (0)	180,000,000..... (∞) 30,000,000..... (2) 5,000,000..... (0)
70	3	150	500 million	5	200,000,000..... (∞) 40,000,000..... (0)	200,000,000..... (0)
70	4	47	30 million	3	16,000,000..... (∞) 3,000,000..... (100) 640,000..... (100) 130,000..... (10) 25,000..... (0)	16,000,000..... (numerous) 3,000,000 (fairly numerous) 640,000..... (0)
71	4	47	400 million	3	80,000,000..... (∞) 16,000,000..... (0)	80,000,000..... (0)

71	5	16	5,000 million	5	80,000,000.....	(0)	80,000,000..... (0)
60	5	12			360,000,000..... (∞)		
					180,000,000..... (500)		
					36,000,000..... (66)		
					7,000,000..... (17)		
					700,000..... (2)		
76	5	35			360,000,000..... (∞)		
					36,000,000..... (500)		
					7,000,000..... (250)		
					700,000..... (20)		
					70,000..... (1)		
71	6	11	8,000 million	5	16,000,000.....	(0)	16,000,000..... (0)
70	8	41			360,000,000..... (∞)		
					180,000,000..... (500)		
					36,000,000..... (141)		
					7,000,000..... (9)		
					700,000..... (1)		

TABLE 5  
*Showing the bactericidal action of normal and paratyphoid-immune rabbit sera upon paratyphoid bacilli*

RABBIT NO.	NUMBER OF PREVIOUS INJECTIONS	INTERVAL SINCE LAST INJECTION	CULTURE USED FOR IMMUNIZATION	CULTURE USED FOR BACTERICIDAL TEST	DOSAGE OF BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF BACILLI SURVIVING IN 1 LOOP OF SERUM AFTER 24 HOURS INCUBATION	APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
39	Normal	<i>days</i>		Paratyphoid A-Groat	360,000 36,000	$\alpha$ 0	36,000 +
81	Normal			Paratyphoid A-5S	160,000,000 26,000,000	4 0	160,000,000
80	Normal			Paratyphoid A-5S	600,000,000 300,000,000 86,000,000 12,000,000	$\alpha$ num. 60 0	86,000,000
15	Normal			Paratyphoid A. Driv.	30,000,000 6,000,000 600,000	num. 3 0	6,000,000
20	Normal			Paratyphoid A. Driv.	70,000,000 14,000,000 1,400,000	$\alpha$ num. num.	?
20+31	Normal			Paratyphoid A. Driv.	82,000,000 20,000,000	2 0	82,000,000
13	Normal			Paratyphoid A. Driv.	100,000,000 50,000,000 25,000,000	f. num. 1 0	50,000,000
13	Normal			Paratyphoid A. Driv.	200,000,000 50,000,000	$\alpha$ num.	10,000,000

19	7	7	Paratyphoid A. Driv.	Paratyphoid A. Driv.	12,000,000 2,000,000	num. 1	2,000,000
19	7	7	Paratyphoid A. Driv.	Paratyphoid A. Driv.	1,400	α	Less than 1,400
19	12	10	Paratyphoid A. Driv.	Paratyphoid A. Driv.	600	α	Less than 600
19	14	10	Paratyphoid A. Driv.	Paratyphoid A. Driv.	1,000 100	α num.	Less than 100
19	14	10	Paratyphoid A. Driv.	Paratyphoid A. Driv.	1,000 100	α 24	100
80	Normal		Paratyphoid B-65	Paratyphoid B-65	220,000,000 36,000,000 6,000,000	α num. 2	6,000,000
14	Normal		Paratyphoid B-65	Paratyphoid B-65	100,000 1,000	0 0	100,000+
			Paratyphoid B. Gach.	Paratyphoid B. Gach.	100,000 1,000 100	α α 0	100
20+31	Normal		Paratyphoid B. Gach.	Paratyphoid B. Gach.	8,000,000 800,000 80,000 8,000	α num. 75 2	8,000
18	Normal		Paratyphoid B. Schott- müller	Paratyphoid B. Schott- müller	14,000 1,000 140	α num. 200	Less than 140
14	Normal		Paratyphoid B. And.	Paratyphoid B. And.	1,500 150	α 0	150
39	15	12	Paratyphoid B. Gach.	Paratyphoid B. And.	16	α	Less than 16

## SUMMARY

The work of Buxton and others showing that normal rabbit serum is capable of killing large numbers of typhoid and paratyphoid bacilli and that the sera of rabbits highly immunized against typhoid and paratyphoid bacilli do not kill these organisms is confirmed; it is emphasized, therefore, that the titer of an immune serum as determined by the usual dilution method with the addition of complement is not an indication of the bactericidal power of the blood plasma *in vivo*.

The bacteriolytic power of normal and highly immune anti-typhoid rabbit serum for typhoid bacilli is neither increased nor decreased by the intravenous injection of a large dose of typhoid bacilli; the serum of a rabbit that has received only one or two immunizing injections shows a distinct, but not a great, increase in its bactericidal power at five and twenty-four hour intervals after such an injection.

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# EXPERIMENTS WITH A POSSIBLE BEARING UPON TREATMENT OF TYPHOID FEVER WITH TYPHOID VACCINE ADMINISTERED INTRAVENOUSLY

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The impossibility of producing typhoid fever in experimental animals renders it difficult to study satisfactorily in the laboratory the mechanism of the cure of typhoid fever patients by means of intravenous injections of large doses of typhoid vaccine. Numerous clinical observations have established the fact that often within twenty-four hours after such an injection the patient ceases to suffer from the typhoid toxæmia. The following experiments were planned to determine whether a rabbit that had received a dose of typhoid vaccine twenty-four hours previously was more resistant to a large dose of typhoid bacilli than a normal control rabbit. If this were the case, it seemed not unlikely that the factor or factors causing the increased resistance in the rabbit were of a similar nature to those bringing about the cure of the typhoid patient.

Rabbits of nearly the same weight were selected for each experiment. One rabbit received an intravenous injection of typhoid bacilli heated one-half hour at 60°C.; it was planned to give the largest dose that would not weaken the rabbit. Twenty-four hours later this rabbit and the control were each injected with 1 cc. of the same suspension of living typhoid bacilli. The results of these experiments are given in table 1.

It was to be expected that normal rabbits would vary in their susceptibility to intravenous injections of typhoid bacilli, and, if this fact is borne in mind, the results are quite decisive and

convincing. In the first four experiments the control rabbits died in 1, 2, 2, and 3 days respectively, while the treated rabbits survived the same doses of living typhoid bacilli. In Ex-

TABLE 1

EXPERIMENT NO.	RABBIT NO.	WEIGHT	DOSAGE FOR PRELIMINARY INJECTION OF TREATED TYPHOID BACILLI	DOSE OF LIVING TYPHOID BACILLI	DIED AFTER	DAY* OF LAST POSITIVE BLOOD CULTURE RECKONED FROM INJECTION
		<i>grams</i>				
I.....	7	2180	Control	Large dose	24 hours	
	54	2095	500 million	Large dose	Survived	
II.....	56	2380	Control	Large dose	40 hours	
	57	2250	100 million	Large dose	16 days	8
III.....	80	2130	Control	Large dose	50 hours	2
	82	1820	100 million	Large dose	Survived	3
IV.....	53	2660	Control	15,000 million	3 days	3
	52	2620	100 million	15,000 million	Survived	3
V.....	51	1620	Control	15,000 million	6 hours	
	49	1300	50 million	15,000 million	1½ hours	
VI.....	42	2605	Control	23,000 million	7 days	7
	45	2240	100 million	23,000 million	2 days	2
VII.....	43	3090	Control	20,000 million	1 day	
	40	3005	150 million	20,000 million	3 days	3
VIII.....	37	2205	Control	10,000 million	11 days	6
	34	2135	Control	10,000 million	3 hours	
	35	2185	100 million	10,000 million	Survived	6
	38	2135	100 million	10,000 million	Survived	4
IX.....	29	2170	Control	12,000 million	Survived	7
	30	2100	100 million	12,000 million	7 days	7
X.....	22	2220	Control	12,000 million	24 hours	
	23	2215	Control	12,000 million	9 hours	
	26	2220	100 million	12,000 million	Survived	3
	24	1916	100 million	12,000 million	Survived	2

\* The blood cultures in bile were taken at irregular intervals only. Rabbits 26 and 24 were negative on the third and fourth days respectively. Rabbit 52 was negative on the sixth day, no. 82 on the tenth day, no. 35 on the seventh day, no. 38 on the fifth day and no. 30 on the eleventh day.

periment V the rabbits were younger and the dose of living typhoid administered was too large, causing the death of both rabbits in a few hours. Experiments VI, VII, and IX, showed no increased resistance in the treated rabbits; but Experiments

VIII and X, each with two controls and two treated animals showed very clearly the increased resistance due to the injection of the vaccine, all four treated animals having survived, while three of the four control rabbits died within twenty-four hours. Young rabbits are more susceptible to typhoid than older ones, and since we did not know the ages of the animals received from dealers, differences in age may have been responsible for some of the irregularities in our results. The heavier rabbit was in every instance selected for the control; in Experiment VI the difference of 350 grams in weight may have obscured the increased resistance caused by the vaccine. We believe that the above experiments justify the conclusion that a preliminary intravenous injection of killed typhoid bacilli confers within twenty-four hours an increased resistance in rabbits to the intravenous injection of a large dose of living typhoid bacilli.

What brings about this increased resistance in the treated animal? In the hope of throwing some light upon this question, the number of bacteria in the peripheral blood was determined at intervals after the injection, the temperature of the animals was measured, and the influence of the preliminary injection upon the number of leucocytes in the peripheral blood was studied.

The temperatures of the rabbits were taken at short intervals following the injection of the living typhoid bacilli in experiments VIII and X and are recorded in table 2.

In Experiment VIII the treated rabbits showed a much more marked rise in temperature than the controls, but in Experiment X the differences are slight. Extreme temperatures ( $106\frac{1}{2}$  and  $106$ ) were reached in rabbits 35 and 38 and were followed by recovery. One of the characteristic features of the treatment of typhoid patients by the intravenous injection of vaccine is the alarming rise in temperature that precedes the drop to normal.

One of us (McWilliams, *Journal of Immunology*, ii, 159) has studied extensively the leucocytosis following the intravenous injection of typhoid bacilli in rabbits and has found that the

injection of 100 million killed bacilli—the dose usually employed in these experiments—causes, as a rule, only a slight leucocytosis in the course of the succeeding twenty-four hours. A few leucocyte counts were made in the first four experiments, in which the treated animals all survived and the controls died in three days or less.

It is seen from table 3 that two of the treated rabbits had a well marked leucocytosis at the time of the test injection, but two other treated rabbits did not show a leucocytosis and were

TABLE 2

*Showing the temperature of the rabbits at intervals after the test injection*

TIME AFTER INJECTION OF LIVING TYPHOID BACILLI	EXPERIMENT VIII				EXPERIMENT X			
	Rabbit 37, control	Rabbit 34, control	Rabbit 35, treated	Rabbit 38, treated	Rabbit 22, control	Rabbit 23, control	Rabbit 26, treated	Rabbit 24, treated
Before injection.....	102 $\frac{1}{5}$	102	103 $\frac{2}{5}$	103 $\frac{1}{5}$	103	103 $\frac{1}{5}$	103 $\frac{1}{5}$	102 $\frac{4}{5}$
1 hr.....	102 $\frac{1}{5}$	101	104 $\frac{2}{5}$	104 $\frac{1}{5}$	104 $\frac{3}{5}$	105 $\frac{1}{5}$	104 $\frac{4}{5}$	103 $\frac{2}{5}$
2 hrs.....	101 $\frac{2}{5}$	99 $\frac{3}{5}$	105	104	105 $\frac{2}{5}$	105 $\frac{1}{5}$	105	104 $\frac{4}{5}$
3 hrs.....	103 $\frac{2}{5}$	97 $\frac{1}{5}$	106 $\frac{1}{5}$	106	105 $\frac{1}{5}$	105 $\frac{2}{5}$	105 $\frac{4}{5}$	105 $\frac{3}{5}$
4 hrs.....	103 $\frac{2}{5}$	Dead	106 $\frac{1}{5}$	105 $\frac{1}{5}$	105 $\frac{3}{5}$	104 $\frac{3}{5}$	105 $\frac{4}{5}$	105 $\frac{4}{5}$
5 hrs.....	103 $\frac{1}{5}$		105 $\frac{1}{5}$	104 $\frac{1}{5}$	104 $\frac{2}{5}$	103 $\frac{1}{5}$	104 $\frac{1}{5}$	105
10 hrs.....	103 $\frac{1}{5}$		105 $\frac{1}{5}$	105 $\frac{1}{5}$	105 $\frac{2}{5}$	Dead	105 $\frac{2}{5}$	105 $\frac{1}{5}$
24 hrs.....	103		103 $\frac{2}{5}$	105 $\frac{1}{5}$	Dead		104 $\frac{1}{5}$	105 $\frac{4}{5}$
48 hrs.....	102 $\frac{1}{5}$		102 $\frac{1}{5}$	102			102 $\frac{4}{5}$	103 $\frac{1}{5}$
3 days.....							102 $\frac{1}{5}$	103 $\frac{2}{5}$
4 days.....	98 $\frac{2}{5}$		104 $\frac{1}{5}$	103 $\frac{1}{5}$				
5 days.....	97 $\frac{1}{5}$		105 $\frac{1}{5}$	102 $\frac{1}{5}$				
6 days.....	100 $\frac{2}{5}$		104 $\frac{1}{5}$	103 $\frac{1}{5}$				
8 days.....	100 $\frac{2}{5}$		103 $\frac{2}{5}$	102				

nevertheless protected. It would seem, therefore, that the increased resistance in the treated rabbits is not due to a leucocytosis called forth by the preliminary injection of the killed typhoid bacilli.

Table 4 demonstrates that the number of viable typhoid bacilli in the peripheral blood rapidly diminishes during the first hour following the injection, after which there seems to be an increase in the control rabbits, while the decrease continues progressively in the treated rabbits. After a few fur-

TABLE 3

Showing the number of leucocytes per cubic millimeter before and 24 hrs. after the preliminary injection

EXPERIMENT NO.	RABBIT NO.	NUMBER OF LEUCOCYTES PER CUBIC MILLIMETER BEFORE THE PRELIMINARY INJECTIONS	NUMBER OF LEUCOCYTES PER CUBIC MILLIMETER BEFORE THE INJECTION OF LIVING TYPHOID BACILLI
I.....	7 control	7,300	12,200
	54 treated	7,000	24,200
II.....	56 control	8,700	10,500
	57 treated	9,000	10,300
III.....	80 control		7,400
	82 treated		9,000
IV.....	53 control	9,200	9,000
	52 treated	10,600	29,600

TABLE 4

Showing the number of colonies developing from three drops of blood plated in agar at various intervals after the injection of living typhoid bacilli in normal and treated rabbits

TIME AFTER INJECTION OF LIVING TYPHOID BACILLI	EXPERIMENT IV		EXPERIMENT VIII				EXPERIMENT X			
	Rabbit 53, control	Rabbit 52, treated	Rabbit 37, control	Rabbit 34, control	Rabbit 35, treated	Rabbit 38, treated	Rabbit 22, control	Rabbit 23, control	Rabbit 26, treated	Rabbit 24, treated
1/4 hr.....	num.	num.								
1/2 hr.....	91	141								
1 hr.....	30	24	49	105	num.	160	89	276	119	66
2 hrs.....	124	42+	72	51	240	178	246	459	91	39
3 hrs.....	205+	52+	37		103	50	102	57	59	37
4 hrs.....			118	Dead	19	16	72	18	20	20
5 hrs.....	56	11	158		11	5	47		4	8
10 hrs.....	15	6	213		15	13	44	Dead	14	24
24 hrs.....	25+	11+	128		19	5	Dead		4	4
48 hrs.....	25	14	72		4	0			1	3
3 days.....	Dead	0*							0	6
4 days.....			2		0*	4			0	0*
5 days.....			2		0*	0				
6 days.....			0*		0*	0				
8 days.....			0		0	0				
11 days.....			Dead		0	0				
15 days.....					0	0				

\* 3 cc. blood in bile positive for typhoid bacilli.

ther hours there is a second rapid decrease in the control rabbits, so that the bacteria are present in the blood in small numbers only at the time of death. In Experiment IV the treated rabbit also showed the increase at the second and third hours but not in such marked degree as the control rabbit.

#### SUMMARY AND DISCUSSION

The intravenous injection of a suitable dose of killed typhoid bacilli in rabbits causes within twenty-four hours a refractory state, which enables them to withstand a dose of living typhoid bacilli that is fatal to normal rabbits. Since antibodies are not demonstrable until four or five days after an injection of antigen, this is not a process of immunization in the accepted sense of the term.

This sudden increase in resistance to living typhoid bacilli bears a certain resemblance to the cure of typhoid fever patients by crisis following the intravenous injection of typhoid vaccine, the reaction in both instances occurring in about twenty-four hours after the injection of the vaccine, which is followed in both cases by a rapid and often extremely high rise in temperature. This suggests that the same factor or factors may be concerned in the two phenomena.

In the preceding article it was shown that the injection of the doses of vaccine used in these experiments does not increase the bacteriolytic power of normal rabbit serum; hence the increased resistance in the treated rabbits is not due to this factor. Nor is it due to an increase in the number of leucocytes in the peripheral blood resulting from the preliminary injection of vaccine; for rabbits that do not show the leucocytosis are also refractory.

The bacteria disappear from the peripheral blood only slightly more rapidly in the treated than in the control animals, there being a rapid decrease in both normal and treated rabbits during the first hour. In the treated animals this decrease continues progressively until the bacteria are very scarce; in the normal controls it appears to be followed by an increase for a

short time and then a rapid decrease until the bacteria are very scarce as in the treated rabbits. The cause of this difference in the normal and treated rabbits is not clear but the evidence at hand indicates that it is not due to an increase of the bacteriolytic power of the blood plasma of the treated rabbits.

For the present we wish merely to record the fact that a rabbit becomes more resistant to infection with typhoid bacilli within 24 hours after the intravenous injection of typhoid vaccine and to point out a possible relationship between the phenomenon and the cure of typhoid fever patients by means of the intravenous injection of typhoid vaccine, without attempting to set up any hypothesis as to the mechanism of the process.





# THE BACTERIOLYTIC POWER OF NORMAL HUMAN SERA AND TYPHOID PATIENTS' SERA FOR TYPHOID BACILLI AND AN INQUIRY INTO THE THEORETICAL BASIS FOR THE TREATMENT OF TYPHOID FEVER WITH VACCINE ADMINISTERED INTRAVENOUSLY

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The bacteriolytic action of the sera of typhoid patients has been studied extensively by two methods: first, by determining the greatest dilution of the serum that will kill a given dose of typhoid bacilli and secondly, by determining the greatest dilution of the serum that, with the addition of rabbit or guinea pig complement, will kill a given dose of typhoid bacilli. By both of these methods the titers of the patients' sera were found to be higher than those of normal human sera; with the addition of complement the patients' sera sometimes killed typhoid bacilli at a dilution of 1/500,000 or even 1/1,000,000. Korte and Steinberg (1) say that the (bacteriolytic) amboceptor content of the serum rises during the first weeks of the disease and falls towards convalescence. Denison (2) obtained similar results. The latter found that complement remains constant throughout the disease no matter what spectacular increase or decrease the immune bodies might show. The patients' sera often exhibited the Neisser-Wechsberg phenomenon, killing with the addition of complement better at higher dilutions than at low ones.

The determination of the amboceptor content of the serum does not necessarily furnish an index of how the undiluted serum or the blood plasma *in vivo* would act upon bacteria. We there-

fore thought it advisable to investigate a number of normal human sera and of typhoid patients' sera by means of the technique used in the preceding article (3).

In our preceding study (3) of the bacteriolytic action of normal rabbit sera, it was found that nearly half of the sera examined killed a million or more typhoid bacilli per cubic centimeter and that most of the others, while not killing these large doses, nevertheless showed a marked bacteriolytic action. Since complement is present in larger and more constant amounts in human serum than in rabbit serum, it was anticipated that normal human sera would kill even larger numbers of typhoid bacilli than normal rabbit sera and this has been proved to be the case.

The non-typhoid human sera were obtained from patients or convalescents in the Hoffman Island Hospital, most of whom had normal temperatures at the time the blood was drawn; the only patient with a high temperature ( $104.8^{\circ}$ ) was No. 7.

The blood was allowed to clot at room temperature and kept in the ice-box over-night. On the following morning the serum was pipetted off and centrifugated, and its bacteriolytic power for typhoid bacilli was determined by exactly the same method that was used for the rabbit sera. It is seen from table 1 that 5 of the normal human sera killed 100 million or more typhoid bacilli per cubic centimeter, 8 killed between 10 and 100 millions and 3 killed between 1 and 10 millions per cubic centimeter. In some instances the largest dose of typhoid bacilli that we employed was completely killed, so that the maximum was not determined; hence the figures should be somewhat higher than these just cited. It is thus apparent that the sera of patients with diseases other than typhoid fever uniformly kill enormous numbers of typhoid bacilli.

We next investigated with the same technique sera of typhoid patients at various stages of the disease and during convalescence. We are greatly indebted to Dr. Richard A. Shields, resident physician at the Hoffman Island Hospital, for drawing the blood for these experiments. Two of the samples of blood were kindly furnished by Dr. Warren Coleman from Bellevue Hospital and one by Dr. Richard Weil from Mt. Sinai Hospital.

TABLE 1

*Showing the bacteriolytic power of normal human sera for typhoid bacilli*

PATIENT NO.	NAME AND AGE AND SEX	DIAGNOSIS	DOSE OF BACILLI PER CC. OF SERUM	NUMBER OF BACILLI SURVIVING IN 1 LOOP OF SERUM AFTER 24 HOURS INCUBA- TION	APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
1	Eri., 23 yrs., male	Beri beri	10,000,000 1,000,000 100,000 10,000	16 14 1 0	10,000,000
2	Ros., 22 yrs., male	Beri beri	40,000,000 20,000,000 1,000,000 100,000	num. f. num. few 0	1,000,000
3	Oli., 26 yrs., male	Beri beri	50,000,000	0	50,000,000+
4	J. And., 21 yrs., male	Bronchitis	65,000,000	0	65,000,000+
5	Nei., 22 yrs., male	Dysentery	10,000,000	0	10,000,000
6	Pan., 45 yrs., male	Bronchitis	60,000,000 12,000,000 1,200,000	$\infty$ num. 0	1,200,000
7	Chris., 34 yrs., male	Relapsing fever	50,000,000 10,000,000	f. num. 0	10,000,000+
8	Bum., 16 yrs., male	Fever	50,000,000	0	50,000,000+
9	Dah., 23 yrs., male	Beri beri	20,000,000 50,000,000	0 0	20,000,000+ 50,000,000+
10	Leg., 20 yrs., male		720,000	0	720,000+
11	Luc., 40 yrs., male	Enteritis	10,000,000	0	10,000,000+
12	Ste., 20 yrs., male	Malaria	10,000,000 1,000,000	1 0	10,000,000
13	Sig., 8 yrs., male	Autointoxi- cation	10,000,000	0	10,000,000

TABLE 1—Continued

PATIENT NO.	NAME AND AGE AND SEX	DIAGNOSIS	DOSAGE OF BACILLI PER CC. OF SERUM	NUMBER OF BACILLI SURVIVING IN 1 LOOP OF SERUM AFTER 24 HOURS INCUBA- TION	APPROPRIATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
14	Fer., 30 yrs., male	Autointoxi- cation	40,000,000	0	40,000,000+
			400,000,000	α	
			200,000,000	25	200,000,000
			100,000,000	10	
			50,000,000	2	
			10,000,000	0	
15	Mol., 49 yrs., male	Beri beri	75,000,000	1	75,000,000
			30,000,000	0	
16	A. And., 29 yrs., male	Inguinal Bubo	300,000,000	num.	
			150,000,000	2	150,000,000
			75,000,000	0	
17	McD., 18 yrs., male	Malaria	100,000,000	2	100,000,000
			50,000,000	0	
18	Dag., 20 yrs., male	Autointoxi- cation	300,000,000	α	
			100,000,000	num.	
			50,000,000	17	50,000,000
			10,000,000	0	
19	Mic., 23 yrs., male	Gonorrheal Rheuma- tism	200,000,000	0	200,000,000
			50,000,000	0	
20	Flo., 35 yrs., male	Malaria	50,000,000	α	
			10,000,000	0	10,000,000
21	Cow., 36 yrs., male	Malaria	300,000,000	α	
			150,000,000	0	150,000,000
22	Dia., 20 yrs., male	Bubo	100,000,000	α	
			50,000,000	f. num.	
			25,000,000	10	25,000,000
23	Saf., 23 yrs., male	Observation	150,000,000	α	
			50,000,000	0	50,000,000
24	Tay., 45 yrs., male	Malaria	100,000,000	α	
			50,000,000	f. num.	
			10,000,000	0	10,000,000

Most of the patients in our hospital were foreigners who were unable to speak English; consequently the duration of their illness previous to admission was not ascertained accurately. The stage of the disease can be judged roughly in each case from the data furnished in table 2. Of the 44 samples of blood serum examined, 3 killed 100 million or more typhoid bacilli per cubic centimeter, 20 killed between 10 and 100 millions, 7 killed between 1 and 10 millions, 8 killed between 50,000 and 1,000,000 and 6 killed less than 50,000 per cubic centimeter. Thus 30 of the 44 sera killed 1 million or more typhoid bacilli per cubic centimeter, showing about the same bacteriolytic power as the normal human sera examined by us. Eight of the other typhoid sera killed fewer bacilli than the normal sera, and 6 killed very much smaller doses. Four of the 6 samples showing a great reduction in bacteriolytic power were from the same patient. The sera were plated both after five and twenty-four hour periods of incubation, and the two readings were usually very much alike; those tubes showing only a few bacteria after five hours yielding as a rule none at all after the longer period. Only the twenty-four hour readings are entered in the table.

For the sake of clearness the results with normal and typhoid sera are summarized in table 3.

Through the kindness of Dr. Agnew of the New York State Hospital for the Insane, in Brooklyn, we received blood from three chronic typhoid carriers of long standing. Their sera were subjected to the procedure described above and were found to kill about 1 million typhoid bacilli per cubic centimeter.

The sera of three paratyphoid patients were tested against typhoid and paratyphoid bacilli. The serum of patient No. 802 killed much greater doses of an old laboratory strain of paratyphoid A. than of his own strain. The serum of the other paratyphoid A. patient also killed only comparatively small doses of his own freshly isolated strain.

Before considering the treatment of any disease one should first endeavor to obtain a clear conception of its pathogenesis. Three different views have been held with regard to the pathogenesis of typhoid fever:

TABLE 2

*Showing the bactericidal power of the sera of typhoid patients for typhoid bacilli*

PATIENT'S HOSPITAL NO.	AGE AND SEX	DATE OF TEST	NUMBER OF TYPHOID BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	DATE OF ADMISSION TO HOSPITAL	DURATION OF ILLNESS	HIGHEST TEMPERATURE IN PREVIOUS 24 HOURS	DATE OF RETURN OF TEMPERATURE TO NORMAL	DATE OF DISCHARGE FROM HOSPITAL	TITER OF MACROSCOPIC AGGUTINATION	EXAMINATION FOR TYPHOID BACILLI					
										Blood		Stool		Urine	
										Date	Result	Date	Result	Date	Result
717	25 yrs. male	iv- 9	25,000,000..... (29)	iii-23	10 days	101½	iv-17	v-23	1/4000	iii-24	+	iii-24	++		
			4,000,000..... (0)									iii-28	+++	iii-31	-
		iv-13	220,000,000..... (α)									iii-31	+++	iv- 3	-
			110,000,000 (f. num.)									iv- 3	+++	iv- 9	-
			31,000,000..... (0)									iv- 9	+++	iv-12	-
												iv-12	+++	iv-25	-
		iv-28	50,000,000 (f. num.)									iv-25	++	iv-28	-
			20,000,000..... (0)									iv-28	+		
			40,000,000 (f. num.)									v- 1	++		
			20,000,000..... (200)									v-11	-		
731	9 yrs. female	v-16	8,000,000..... (15)	iv-8	8 days	98½	v-2	v-23	1/10			v-15	-		
			4,000,000..... (2)												
		iv-13	1,000,000..... (0)												
			220,000,000..... (α)												
			110,000,000 (f. num.)									iv- 9	-	iv- 9	-
			31,000,000..... (0)									iv-11	-		
												iv-11	-	iv-15	-
												iv-13	+		
												iv-18	-		



TABLE 2—Continued

PATIENT'S HOSPITAL NO.	AGE AND SEX	DATE OF TEST	NUMBER OF TYPHOID BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	DATE OF ADMISSION TO HOSPITAL	DURATION OF ILLNESS	HIGHEST TEMPERATURE IN PREVIOUS 24 HOURS	DATE OF RETURN OF TEMPERATURE TO NORMAL	DATE OF DISCHARGE FROM HOSPITAL	TITRE OF MACROSCOPIC AGGLUTINATION	EXAMINATION FOR TYPHOID BACILLI			
										Blood		Stool	
										Date	Result	Date	Result
764	18 yrs. male	v-7	8,000,000. .... (0)	v-1	4 days	102	v-20	vii-5	1/20	v-3	—	v-2	+
												v-5	—
		v-24	160,000,000 (f. num.) 40,000,000. .... (4) 8,000,000. .... (0)			98 $\frac{2}{3}$			1/160			v-8	—
												v-10	++
												v-11	++
762	24 yrs. male	v-7	8,000,000. .... (0)	v-1	?	101	v-7	v-23	1/10	v-3		vi-1	—
												vi-10	—
		v-10	100,000. .... (0)	v-3	5 days	101 $\frac{1}{2}$	v-19	vi-26				vi-13	—
												vi-16	—
												vi-11	—
767	23 yrs. male	v-10	100,000. .... (0)	v-3	5 days	101 $\frac{1}{2}$	v-19	vi-26				v-2	+
												v-4	+
		vi-2	47,000,000. .... (1) 19,000,000. .... (0)			98 $\frac{2}{3}$				v-5	+	v-5	+
												v-8	+
												v-12	++
												v-15	+
												v-17	—
												v-29	+
												vi-1	—
												vi-6	++
												vi-8	—
												vi-9	—
													+
													+
													+



768	23 yrs. male	v-23	120,000,000 (f. num.) 60,000,000..... (1) 6,000,000..... (0)	v- 3	10 days	98 $\frac{3}{4}$	v-16	vi-16		v- 3	-	v- 4 v- 8 v-11 v-20 v-25 v-29 vi- 1 vi- 7	++ ++ ++ ++ - - - -	v- 4 v-11 v-20 v-25 v-29 vi- 1 vi- 7	- - ++ ++ ++ ++ - -
800	24 yrs. male	vi- 2	47,000,000..... (0)			98 $\frac{3}{4}$									
		v-12	50,000,000..... (29) 2,000,000..... (0)	vi- 9	3 days	103 $\frac{1}{2}$		Died vi-24	1/320	vi-12	+	vi- 9 vi-13 vi-15 vi-16 vi-20 vi-22 vi-23	- ++ ++ ++ ++ ++ +	vi-13 vi-14	++ ++
826	27 yrs. male	vii-13	50,000,000..... (6) 10,000,000..... (0)	vii-12	8 days	102 $\frac{1}{2}$				vii-13	-	vii-16 vii-18 vii-21 vii-22 vii-28	++ + + - -	vii-17	-
834	17 yrs. female	vii-16	50,000,000 (f. num.) 10,000,000..... (19) 1,000,000..... (1) 100,000..... (0)	vii-16	7 days	103		viii-11	1/640	vii-16	-	vii-17 vii-19 vii-23 vii-24 vii-26 viii- 2	++ + ++ ++ - -	vii-17 vii-19	++ +

TABLE 2—Continued

PATIENT'S HOSPITAL NO.	AGE AND SEX	DATE OF TEST	NUMBER OF TYPHOID BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	DATE OF ADMISSION TO HOSPITAL	DURATION OF ILLNESS	HIGHEST TEMPERATURE IN PREVIOUS 24 HOURS	DATE OF RETURN OF TEMPERATURE TO NORMAL	DATE OF DISCHARGE FROM HOSPITAL	TITER OF MACROSCOPIC AGGLUTINATION	EXAMINATION FOR TYPHOID BACILLI					
										Blood		Stool		Urine	
										Date	Result	Date	Result	Date	Result
884	19 yrs. male	ix-30	720,000..... (α) 72,000.... (num.) 720..... (38) 72..... (7)	ix-18	15 days	103	x-22	xi-10	1/640						
		x-7	600,000..... (68) 60,000..... (36) 12,000..... (0)			101½			1/1280	x-1	—				
903	21 yrs. male	x-12	8,500,000..... (α) 850,000..... (154) 85,000..... (3) 8,500..... (0)			100			1/2560						
		x-4	1,000,000..... (0) 21,000,000..... (0)	x-3	14 days	102½	x-25	xii-1	1/640	x-4	—				
		x-23				100									



TABLE 2—Continued

PATIENT'S HOSPITAL NO.	AGE AND SEX	DATE OF TEST	NUMBER OF TYPHOID BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	DATE OF ADMISSION TO HOSPITAL	DURATION OF ILLNESS	HIGHEST TEMPERATURE IN PREVIOUS 24 HOURS	DATE OF RETURN OF TEMPERATURE TO NORMAL	DATE OF DISCHARGE FROM HOSPITAL	TITEN OF MACROSCOPIC AGGLUTINATION	EXAMINATION FOR TYPHOID BACILLI					
										Blood		Stool		Urine	
										Date	Result	Date	Result	Date	Result
Sin.	30 yrs. male	x-31	45,000,000..... (∞) 22,000,000...(num.) 9,000,000.... (174) 900,000..... (7) 90,000..... (3) 9,000..... (1)	13 days		105		Died xi-13	1/640	x-31	+				
										xi- 2	+				
921	23 yrs. male	x-30	50,000,000..... (0)	x-28	10 days	102	xi-10	xi-23	1/640	xi- 2	-	x-29	++	x-29 x-30	- +
												xi- 2	++	xi- 5	-
												xi- 7	++	xi-10	-
												xi-20	-	xi-20	-
							xi-3	xii-27	1/1280			xi- 6	-	xi- 6	-
												xi- 7	-	xi- 7	-
						103%						xi- 8	++	xi- 8	+
												xi-11	++	xi-11	++
												xi-15	++	xi-15	++
												xi-21	+	xi-21	+
												xi-23	-	xi-23	-
												xi-29	-	xi-29	-
												xi-8	-	xi- 8	-
												xii-12	-	xii-12	+
												xii-16	-	xii-16	+
												xii-26	-	xii-26	-
929	23 yrs. female	xi-7	75,000,000..... (0)	xi-4	14 days										

927	13 yrs. male	xi-9	100,000,000..... (0)	xi-2	4 days	100°	xi-10	xi-23	1/1280	xi-9	-	xi-8 xi-9 xi-11 xi-13 xi-15 xi-21	- - + - - -	xi-5 xi-8 xi-9	- - +
Coy	26 yrs. female	xi-14	50,000,000 (f. num.) 25,000,000..... (4) 10,000,000..... (1) 1,000,000..... (0)						1/1280	xi-14	+			xi-13 xi-15 xi-21	+ - -
934	23 yrs. male	xi-23	300,000,000 (f. num.) 100,000,000..... (7) 50,000,000..... (0)	xi-21	4 days	102°	Tuber- culosis	i-5	1/320			xi-22 xi-23 xi-25 xi-30 xii-4 xii-9 xii-11 xii-12 xii-16 xii-26 i-5	- - + - - - - - - -	xi-22 xi-23 xi-25 xi-30 xii-4 xii-9 xii-11 xii-12 xii-16 xii-18 xii-20 i-5	- + - + + + + + + + +
		xii-4	300,000,000..... (α) 150,000,000..... (37) 50,000,000..... (0)			102%			1/160	xii-4	-				+ + + + + +

TABLE 2—Concluded

PATIENT'S HOSPITAL NO.	AGE AND SEX	DATE OF TEST	NUMBER OF TYPHOID BACILLI KILLED PER CUBIC CENTIMETER OF SERUM	DATE OF ADMISSION TO HOSPITAL	DURATION OF ILLNESS	HIGHEST TEMPERATURE IN PREVIOUS 24 HOURS	DATE OF RETURN OF TEMPERATURE TO NORMAL	DATE OF DISCHARGE FROM HOSPITAL	TITER OF MACROSCOPIC AGGLOUTINATION	EXAMINATION FOR TYPHOID BACILLI					
										Blood		Stool		Urine	
										Date	Result	Date	Result	Date	Result
935	25 yrs. male	xi-23	1,000,000..... (α)	xi-21	21 days	103½	xii-27		1/80	xi-23	+	xi-23	++	xi-23	++
			100,000..... (α)							xi-27	+	xi-27	++	xi-27	++
			10,000..... (50)									xi-29	++	xi-29	++
			1,000..... (0)									xi-29	++	xi-29	++
		xi-27	1,000,000..... (α)									xii-2	++	xii-2	++
			100,000.. (num.)									xii-4	++	xii-4	++
			10,000..... (51)									xii-5	+	xii-5	++
			1,000..... (0)									xii-6	-	xii-6	++
	xii-4	10,000,000..... (α)			xii-11	-	xii-11	++							
		1,000,000.. (num.)			xii-16	-	xii-16	++							
		100,000..... (24)					xii-18	+							
		10,000..... (2)					xii-23	+							
		1,000..... (0)					xii-27	-							
		100,000..... (α)					i-3	-							
		10,000..... (43)					i-8	-							
		1,000..... (0)						-							
	i-8		100,000..... (α)			98½		1/160							
10,000..... (113)															
1,000..... 0															

TABLE 3

DOSAGE	NORMAL SERA	TYPHOID SERA
100 million or more.....	5	3
10 million to 100 million.....	18	20
1 million to 10 million.....	3	7
50,000 to 1 million.....		8
Less than 50,000.....		6

TABLE 4

*Showing the bactericidal power of the sera of chronic typhoid carriers for typhoid bacilli*

NAME	DOSAGE OF TYPHOID BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF TYPHOID BACILLI SURVIVING IN ONE LOOP OF SERUM AFTER TWENTY-FOUR HOURS INCUBATION	APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
Gas.....	160,000,000	α	1,000,000
	40,000,000	f. num.	
	1,000,000	8	
	160,000	0	
Loa.....	160,000,000	α	1,000,000
	40,000,000	f. num.	
	1,000,000	0	
Cal.....	160,000,000	α	1,000,000
	40,000,000	f. num.	
	1,000,000	0	

1. It is a blood disease, a typhoid septicemia, and the intestinal lesions are only a localization of the process and are of comparatively secondary importance. The bacteria pass from some point in the intestinal tract, for example from the tonsils, directly into the blood and from there into the lymph glands and lymphatic apparatus of the intestines.

2. It is a primary infection of the intestinal follicles. The mesenteric lymph glands are next infected and finally the typhoid bacilli break through into the blood, where they multiply, producing, as it were, a secondary septicemia.

3. "In typhoid fever the bacillus first finds its way from the alimentary tract to the lymphopoietic system, including the

TABLE 5  
Showing the bactericidal action of sera of paratyphoid patients against typhoid and paratyphoid bacilli

EXAMINATION FOR BACILLI																	
PATIENT NUMBER AND ILLNESS	NAME AND AGE AND SEX	DATE OF TEST	CULTURE USED FOR BACTERICIDAL TEST	NUMBER OF BACILLI KILLED PER CC. OF SERUM AFTER TWENTY-FOUR HOURS INCUBATION	DATE OF ADMIS- SION TO HOSPI- TAL	DURATION OF ILLNESS	HIGHEST TEMPERA- TURE IN PREVIOUS HOURS	DATE OF RETURN OF TEMPERATURE TO NORMAL	DATE OF DISCHARGE FROM HOSPITAL	Blood				Stool		Urine	
										Result		Date		Result		Date	
										Date	Result	Date	Result	Date	Result	Date	Result
709 Paraty- phoid A	Driv., 24 yrs. male	iv-29	Paraty- phoid A, Driv.	50,000,000..... (α) 10,000,000... (num.) 1,000,000 (f. num.) 100,000..... (21) 10,000..... (0)	iii-16	7 days	99°	v-27	v-31	iii-24 +	iii-17 iii-22 iii-24 iv-1 iv-7 iv-15 iv-25 iv-27 v-15	- - + + + + + - -	iii-17 iii-22 iii-24 iv-1 iv-7 iv-15 iv-25 iv-27 v-15	- - + + + + + - -	iv-1 iv-7 iv-15 iv-25 iv-27 v-15	- - - - - - -	
829 Paraty- phoid B	Alg., 6 yrs. female	vii-17	Typhoid Ant.	50,000,000 (f. num.) 10,000,000..... (30) 1,000,000..... (0)	vii-15	5 days	99°	vii-25	ix-8	vii-15 -	vii-21 vii-28 viii-3 viii-9 viii-14 viii-18 viii-25 viii-31 ix-3	+ + - + - + + + + + + + + +	vii-21 vii-28 viii-3 viii-9 viii-14 viii-18 viii-25 viii-31 ix-3	+ + - + - + + + + + + + + -	viii-3 viii-14 viii-18 viii-25 viii-31 ix-3	+ + - - - - -	



802 Paratyphoid A	Gro., 28 yrs., male	vi-13	Typhoid- Ant.	50,000,000.....	(0)	vi- 9	8 days	101 <sup>20</sup>	vi-24	vii -7	vi-12 +	vi- 9 vi-12 vi-15 vi-22	— — — —	vi-15 vi-23	— —
		vi-15	Paratyphoid A-58	500,000,000.....	(0)			101 <sup>21</sup>			vi-21 —				
		vi-21	Paratyphoid A, Gro.	360,000,000..... 36,000,000 (f. num.) 3,600,000 (f. num.) 360,000..... 36,000.....	(α) (f. num.) (f. num.) (8) (0)			101							

spleen, where it develops chiefly and from which it invades the blood stream. We think it doubtful whether the bacillus multiplies in the blood, but rather that its presence there simply represents an overflow from the lymph organs. Under this interpretation the presence of the bacillus in the blood does not constitute a true septicemia" (Buxton and Coleman (4)).

Our experiments and similar ones by other workers show that typhoid bacilli could never multiply in the blood serum of normal persons; hence the first hypothesis, viz., that typhoid fever is a primary septicemia, is untenable. Furthermore the serum of typhoid fever patients is only very exceptionally of low bactericidal power and consequently the view that the disease is a septicemia following the infection of the intestinal follicles and mesenteric glands must be discarded. There remains the third hypothesis, viz., that the disease is a localized infection and that the typhoid bacilli are present in the blood but do not multiply there. Unfortunately no determinations of the number of bacilli in the blood at various stages of the disease have been made, but the fact that it has become general practice to use large amounts of blood (10 to 20 cc.) for enrichment in bile would indicate that usually only a few bacilli are present to the cubic centimeter of blood. In pneumonic plague, which is a true septicemia, several plague bacilli to the field are frequently seen in a hanging drop preparation of the blood.

The recent experiments of Rous (5), showing that typhoid bacilli after ingestion by guinea pig leucocytes are protected against the destructive action of normal rabbit serum, suggest that the typhoid bacilli in the blood of typhoid patients are probably contained within the leucocytes or endothelial cells that have escaped from the spleen or mesenteric glands. This offers a very plausible explanation of the presence of the bacilli in small numbers in the blood of patients throughout the greater part of the disease in spite of the fact that the blood serum is highly bacteriolytic, and is thus in harmony with the view that typhoid fever is not a true septicemia. But the constant presence of a few living bacilli in the highly bacteriolytic blood and the transfer of living bacilli from the mesenteric glands through

the blood to the spleen and bone marrow can be explained without the protective action of the leucocytes; the bacteriolytic action of the serum is comparatively slow, usually requiring a half hour or more for the complete destruction of bacilli; hence the few bacilli that had been in the blood only a few minutes at the time of the bleeding would have suffered but little injury and could multiply when transferred to a favorable culture medium.

Adami (6) says: "What determines the development of symptoms of general infection is not the presence of the specific bacteria circulating throughout the body, or even the extent of the local inflammatory disturbances set up by them, but is the toxicity of their products and the relative amount of the same." In typhoid fever it is not known at present whether these toxic products of the typhoid bacilli are derived solely from free bacilli that are dissolved in the blood stream or whether they are also discharged from the spleen, mesenteric glands and other local lesions into the lymph and blood.

Having arrived at a definite conception of the pathogenesis of the disease, we shall now consider the mechanism of the cure of typhoid fever by means of the intravenous injection of vaccine. This method of treatment is not an immunization in the usual sense of the term, since the result is often obtained within twenty-four hours, whereas the increase in antibodies following the injection of antigen does not occur until after three or four days, even in immunized animals. The patient may become more highly immunized against the typhoid bacillus in the course of the next few days as a result of the injection, but this immunization is apparently not connected with the cure by crisis which we are discussing. A number of hypotheses have been advanced in explanation of the phenomenon:

1. *Hyperleucocytosis*. It has been shown by Holler (7), Gay (8), McWilliams (9) and others that the intravenous injection of typhoid vaccine in typhoid patients calls forth a high-grade hyperleucocytosis and this increase of leucocytes in the peripheral blood has been thought by Löwy, Luksch and Wilhelm (10) and others to bring about the cure of the patient.

Typhoid fever has also been reported cured by crisis following the injection of killed *B. coli* (Kraus (11), of deuterio-albumose (Lüdke (12) ) and of colloidal gold (Letulle and Mage (3), Gay (14) ). Since all of these substances likewise call forth a hyperleucocytosis it was considered that the treatment with typhoid vaccine is entirely non-specific and is due, as in these instances, to the hyperleucocytosis alone. Gay believed that his typhoid vaccine called forth a more marked leucocytosis than these other substances and that the benefit from the treatment was due to the combination of the hyperleucocytosis and the presence of antibodies in the patient's blood. He also says, "Typhoid vaccine aids in building up the active immunity of the patient against typhoid fever as no other preparation can," but this obviously refers to the prevention of relapses and not to the mechanism of the cure.

This hypothesis is, we believe, untenable, because the blood serum itself without the assistance of the leucocytes is, as we have seen, well able to destroy the bacilli that enter the blood in typhoid fever. A modification of the hypothesis, viz., that the leucocytes by ingesting and destroying the bacilli within their bodies render inert the toxic bacillary products that would otherwise be set free in the circulation, appears to us somewhat more plausible. However, the hyperleucocytosis is only temporary and the bacteria proliferating in the tissues should after its subsidence be able to enter the circulation and call forth toxic symptoms as before.

2. *Paralysis of the heat center.* The injection of vaccine is usually followed in a short while by a very high temperature. Paltauf (15) suggested that this over-stimulation might lead to a paralysis of the heat center of the central nervous system so that toxic bacillary substances in the circulation would then fail to cause fever. He disproved his own hypothesis, however, by showing that a malarial patient still developed a high malarial temperature after the typhoid vaccine had been administered.

3. *Rapid mobilization of antibodies.* Bull (16) found that the intravenous injection of a large dose of typhoid vaccine caused no drop in the concentration of the antibodies in the serum but,

on the contrary, in some instances brought about an increase in certain of these in from five to twenty-four hours after the injection. He suggested that such a rapid mobilization of antibodies might possibly be responsible for the cure of typhoid patients by the intravenous injection of vaccine. In a preceding article we have shown that the bacteriolytic antibodies in immune rabbits are increased to a slight degree only in twenty-four hours after the injection of large doses of vaccine; since the sera of typhoid patients are already strongly bacteriolytic, one is not justified in supposing that a slight increase in this property would cause a radical change for the better in the condition of the patient.

4. *Stimulation of non specific ferments.* Jobling and Petersen (17) injected a dog with dried typhoid bacilli in a sufficiently large dose to cause death in seven hours and found an increase in both the protease and lipase of its serum. Later Jobling (18) stated that in patients a similar reaction occurs following the intravenous injection of vaccines and proteoses, but not to the same degree nor with the same regularity as in animals. It was suggested that the cure of typhoid patients might be brought about after the intravenous injection of vaccine by the mobilization of these non-specific ferments; the protease might produce a more rapid splitting of the toxic protein fragments derived from the typhoid bacilli to lower non-toxic forms, while an increase in the lipolytic ferments may have some influence in destroying the living typhoid bacilli.

There seems to be, as yet, very little experimental basis for this hypothesis.

5. *Anti-anaphylaxis.* The sudden and violent reaction following the intravenous injection of vaccine in typhoid patients and the rapid recovery suggested early that the phenomenon might in some way be related to anaphylactic shock and that the patient after the reaction might be in a condition of anti anaphylaxis with regard to the typhoid proteins. The hypothesis that fever (and perhaps certain other symptoms) in infectious diseases is due to repeated anaphylactic reactions has been much discussed. It is thought that the patient becomes sensitized

to the proteins of the infecting microörganism and that as the latter enters the circulation, repeated low grade anaphylactic attacks are called forth without producing complete anti-anaphylaxis. The sudden introduction of a large amount of antigen might, under these circumstances, completely desensitize the patient. The site of the anaphylactic reaction has been shown to be mainly, if not entirely, in the cells and not in the blood stream; hence when Gröer (19) assumes that the intravenous injection of vaccine brings about a failure of the body cells to react to the typhoid toxins or proteins, or a condition similar to the "immunity through lack of receptors" he is merely setting forth the conception of anti-anaphylaxis in different terms.

Ichikawa (20) found that paratyphoid patients as well as typhoid patients were cured by the injection of typhoid vaccine and Kraus (11) found that colon vaccine cured typhoid patients in the same way as the typhoid vaccine; Kraus therefore claimed that the reaction is not an anaphylactic one, since it is not specific.

Aside from the objection of Kraus, the anti-anaphylaxis hypothesis is not entirely satisfactory; for the bacilli should continue to multiply in the local lesions and after a time re-sensitize the patient and cause a return of the fever.

None of the above hypotheses seems to us to offer an adequate explanation of the phenomenon under discussion. We wish to suggest the following explanation, which we think is more plausible than those discussed above, realizing, however, that its proof will be most difficult.

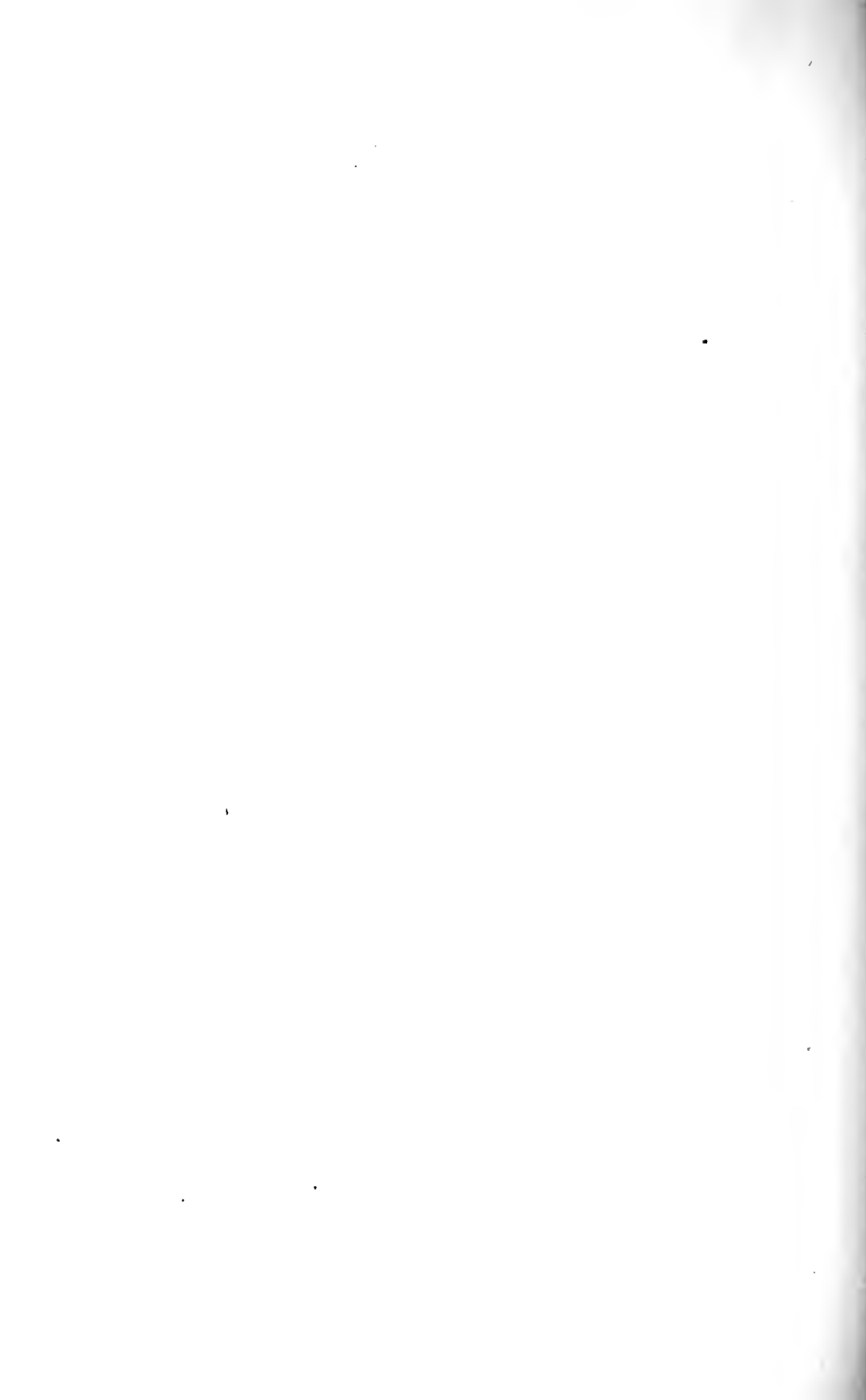
We believe that typhoid fever is a local disease and not a septicæmia; therefore the curative process must take place at the site of the local lesions. Hughes and Carlson (21) and Becht and Greer (22) showed that in both normal and immune animals the antibody concentration is greater in the blood serum than in the thoracic lymph. We have seen that in almost all cases of typhoid fever the serum is strongly bacteriolytic for typhoid bacilli throughout the disease; it would seem that the bacteria multiply in certain tissues because the tissue fluid or lymph does not possess this property. If the intravenous injection of vaccine should cause a more active passage of bacteriolytic

substances from the blood capillaries into the lymph the destruction of the typhoid bacilli and healing of the local lesions should be effected. This is the hypothesis we wish to suggest.

This explanation might also explain the survival of the treated rabbits in the preceding article (23). The serum of normal rabbits is usually strongly bacteriolytic for typhoid bacilli; the intravenous injection of the large dose of vaccine probably causes more of the bacteriolytic substances to pass from the blood capillaries into the lymph, so that on the following day the bacteria lodging in the spleen and liver find conditions unfavorable for growth.

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## HAY FEVER

### THE NATURE OF THE PROCESS AND OF THE MECHANISM OF THE ALLEVIATING EFFECT OF SPECIFIC TREATMENT

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Since the etiological relation of the pollens of certain plants to hay fever and some forms of asthma was established, a number of investigators have studied the question of the nature of the active substances in the pollens, as well as the mechanism of the curative or alleviating effect of the two forms of specific treatment (with "antitoxin"—"pollantin" of Dunbar—and with active immunization as first practised by Noon).

Originally, both of these methods of treatment were based upon the assumption that the active substances of the pollens are toxins (toxalbumins) to which the former method induced a passive immunization, while the latter, as already indicated, caused an active immunization by the production of specific antibodies.

This conception has been supplanted by the theory of Wolff-Eisner (1),<sup>1</sup> according to which the clinical manifestations of hay fever are expressions of hypersensitiveness to an otherwise non-toxic constituent of the pollens. Dunbar's assumption of a toxin nature for the active substances of the pollens is based, first, upon the injurious effect of those substances on susceptible persons, and, secondly, upon his belief that he had succeeded in producing a specific antitoxin to the substances by the usual method of immunization.

<sup>1</sup> Koessler attributes this theory in part to Weichardt. However, a review of the literature fails to substantiate Weichardt's participation in the theory.

The chief objection to the assumption of a toxin nature on the part of the active substance of the pollens lies in the fact that, to the great majority of individuals, as many as a thousand times the amount of the pollen extracts that is toxic for the hay fever patient is entirely innocuous.

Such resistance to a true toxin would amount to an absolute immunity, such as does not exist in individuals of any species of animals that is susceptible to the toxins. This non-susceptibility of normal individuals to the pollens is comparable to the natural relative resistance of certain persons to diphtheria toxin. However, as Cooke and Vander Veer (2) have pointed out, the difference between these two conditions is shown in the fact that whereas, in the latter instance, the resistance has been shown always to depend on the presence of natural antitoxin in the body fluids of the relatively immune individuals, in the former instance no such antibodies have been demonstrated.

An important requirement of a toxin is that its injection into animals be followed by the production of an antitoxin and, as we have said, this requirement is claimed by Dunbar to have been fulfilled for grass pollen by his patented preparation "Pollantin." However, Dunbar's claim in this respect has been refuted by Wolff-Eisner (3), who discussed two main objections to it. These were: first, that the curve of saturation of pollen extract and "Pollantin," as plotted by Dunbar's assistant, Prausnitz (4) is completely out of harmony with the law of multiple proportions, which governs the neutralization of all known toxins and their specific antitoxins; and, secondly, that the alleged antitoxin of Dunbar is toxic to hay fever patients when injected subcutaneously. To these may be added a third objection, namely, that normal sera of cattle, as Weichardt showed, have a therapeutic effect at least equal to that of "Pollantin."

Dunbar's method of determining the presence of specific antibodies in his "Pollantin" was at first entirely therapeutic, both the precipitin and the complement-fixation method failing in his hands. Later, in 1908, he states (5) that he succeeded in demonstrating antibodies with the latter of the methods referred

to.<sup>2</sup> The value of the therapeutic test is at least placed in suspicion by the observation of Weichardt that a similar effect is obtained with the serum of normal animals, taken in the summer season, and Dunbar's results with the *in vitro* tests are not in accord with our own, which were obtained during attempted immunization of two rabbits with strong extracts of the pollen of ragweed (*Ambrosia trifida*) and of redtop (*Agrostis alba*).

The course of treatment of the rabbits was as follows: for the injections, simple extracts of the pollens were made with 0.8 per cent of NaCl and to these was added 0.25 per cent trikresol. The ragweed pollen extracts R1 contained 1.34 mgm. of nitrogen per cubic centimeter, the redtop pollen extract RT8 contained 0.55 mgm. of nitrogen per cubic centimeter. Between June 5 and July 17 Rabbit 208 received 28 intravenous injections of RT8 and Rabbit 77 received an equal number of injections of R1. The injections were made on the first, fifth, sixth, ninth and thirteenth days and daily thereafter, excepting Sundays. For the first five injections, 1 or 2 cc. of R1 were used and 2 or 4 cc. of RT8. Thereafter these quantities were reduced to 0.4 cc. and 0.8 cc. respectively. The plan of daily injections was adopted because of the uniformly superior results obtained with this method by Elser and Huntoon (7) with meningococcus and by Coca with red blood corpuscles.

On the twelfth day of the treatment both animals were bled and 0.2 cc. of the active sera was mixed in two series with descending amounts of the corresponding pollen extracts, from 0.2 cc. to 0.003 cc. After twenty-four hours at room temperature no precipitation had taken place in any of the mixtures. Similar series, to each tube of which was added also 0.5 cc. of fresh guinea pig's serum (complement), were prepared with the inactivated rabbit's sera in order to test these sera for complement fixation bodies. However, no trace of specific fixation was observed in any of the mixtures. All of these experiments were

<sup>2</sup> Clowes,<sup>6</sup> also, using pollen extracts as antigen, reports precipitin and complement fixation reactions in certain—not all—cases before the commencement of the hay fever season. These reactions disappeared under treatment, reappearing a few weeks later.

exactly controlled with similar series carried out with two normal rabbits' sera.

The blood of the treated rabbits was again examined on the twenty-second day, on the thirty-first day and on the forty-fifth day, but on none of these occasions were the antibodies that were sought for found.

A third rabbit, No. 262, received thirteen injections of 0.8 cc. of RT8 and its serum was examined as described above, on the third day after the last injection, also with completely negative results.

These consistently negative results are in agreement with Weichardt's adverse opinion of Dunbar's claim that his "Pollantin" represents a specific "antitoxin." However, since the reactions of specific precipitation and of complement fixation are less reliable methods of demonstrating antibody production in injected animals than is the anaphylactic reaction we have conducted a series of experiments with the object of actively sensitizing guinea pigs to ragweed pollen extract. *With this sensitive and reliable reaction, also, the attempt to demonstrate antibody production after the injection of pollen extract failed entirely.* The manner in which the anaphylactic reaction was applied is shown in table 1.

Dunbar (8) reports the active sensitization of 14 out of 21 guinea pigs that had received injections of rye pollen. Since there is no reason to assume any difference in the antigenic properties of the different pollens we are unable to offer any explanation of these contradictory observations. Dunbar's experiments are described without protocol.

The failure of Dunbar's claim regarding the specific quality of his "Pollantin" removes the strongest support of the assumption of a toxin nature of the active pollen substances. In fact, this assumption may be said to be left without any support.

When we examine the grounds of the alternative theory of Wolff-Eisner, that of the anaphylactic nature of the hay fever process, we find that the theory is supported chiefly by the fact that the afflicted individuals are sensitive in a more or less high degree to the administration, local or general, of a substance

which, to at least 90 per cent (9) of all persons, is entirely innocuous in a 500- or 1000-fold dose. Indeed the hypersensitivity may be said to be self-evident in this statement of the unchallenged fact.

It is proper to inquire whether either of the two other characteristic phenomena of the anaphylactic state is present in hay fever. We refer to the phenomenon of anti-anaphylaxis or desensitization and that of passive transfer of the hypersensitivity.

TABLE 1

*Attempted active sensitization of guinea pigs with extract of ragweed pollen*  
(1 cc. = 1.34 mgm. N.)

GUINEA PIG	PRIMARY INJECTION	INTERVAL	INTRAVENOUS TEST INJECTION	RESULT
	<i>cc.</i>	<i>days</i>	<i>cc.</i>	
202	0.05	17	1.0*	No symptoms
208	1.0	18	1.0	No symptoms
254	2.0	21	1.0	No symptoms
255	2.0	21	1.0	No symptoms
257	2.0	21	1.0	No symptoms
258	2.0	21	1.0	No symptoms
259	2.0	21	1.0	No symptoms
260	2.0	21	1.0	No symptoms
217	1.0	68	1.0	No symptoms
209	1.0	74	1.0	No symptoms

\* Calculated upon the basis of relative protein content (compared with horse serum) this quantity of the ragweed extract should represent about 13 minimal doses.

Next to the anaphylactic shock, the condition of desensitization is the most striking phenomenon of the state of hypersensitivity, and it can generally be induced by the appropriate injection of a sublethal quantity of the antigen. Since it has been found impossible experimentally to sensitize animals to the pollens, we must confine our study of the phenomenon of desensitization in the human sensitizations to the pollens, to human subjects.

It has, in fact, been found possible by many observers (10), to render hay fever subjects resistant to the local action of the

respective pollens. This condition of resistance is distinguished from that of experimental desensitization in animals by the following features: it is generally established only after a number of injections of the specific pollen, instead of after a single injection; it is established with fewer injections and after a shorter time in some cases than in others, the more susceptible cases yielding more readily to the treatment.

The necessity of making a number of injections in rendering the hay fever subject resistant has an obvious explanation. It is due to the fact that the amount of the pollen extract that may be injected at one time is limited on account of the unpleasant symptoms which follow the administration of larger doses.

The reason for the differences among hay fever patients with respect to the effect of the pollen injections can only be surmised. If the resulting resistance is due to a gradual saturation or neutralization of an antibody-like substance with the active pollen substance, we may assume that in the less susceptible individuals the union of these two bodies is a much less firm one than that in the more susceptible individuals and that the active pollen substance is discharged from such a combination and eliminated much more quickly in the former than in the latter. Such a phenomenon finds an analogy in the temporary binding of complement observed by von Dungern and Coca (11) when chicken serum and its rabbit antiserum were united in the presence of active guinea pig's serum. There was at first a complete fixation of the complement, which afterward was gradually discharged until, at the end of twenty-four hours, it was demonstrable in its full original activity in the mixture.

In view of the demonstrated fact that guinea pigs can not be rendered anaphylactic by injections of ragweed pollen extract and in view of our failure to obtain immune bodies by the injection of rabbits with pollen extracts, it seemed futile to attempt the sensitization of guinea pigs by the method of passive transfer. However, since Koessler (12) (without protocol) has claimed to have applied this method successfully, we have carried out a few similar experiments.

Koessler states that he injected the serum of two hay fever patients into guinea pigs in a dose of 4 cc. On the following day he "reinjects" the animals, all of whom "showed severe typical symptoms of anaphylactic shock."

We injected the fresh unheated citrate plasma of ten hay fever subjects, intravenously, in a dose of 6 cc. into ten guinea pigs. The test injections were made intravenously at different intervals as indicated in table 2. In no instance were the test injections followed by any toxic symptoms.

TABLE 2

*Attempted passive sensitization of guinea pigs to pollen extracts*

CASE	YEARS TREATED	INTERVAL SINCE LAST INJECTION	TYPE OF HAY FEVER	AMOUNT OF SE- RUM INJECTED	POLLEN EXTRACT INTRAVENOUSLY	INTERVAL	RESULT
				cc.		hours	
495	1	1 month	Late	6.0	R1, 1.0 cc.	48	No symptoms
46	?	Months	Early and late	6.0	RT, 1.0 cc.	48	Twitchings
					R1, 1.0 cc.*	48	No symptoms
435	1	Months	Early and late	6.0	R1, 1.0 cc.	48	No symptoms
					RT, 1.0 cc.	48	No symptoms
14	3	1 month	Late	6.0	R1, 1.0 cc.	72	No symptoms
341	1	Months	Early	6.0	RT, 1.0 cc.	72	No symptoms
96	3	Months	Early	6.0	RT, 1.0 cc.	24	No symptoms
192	2	2 days	Early	6.0	RT, 1.0 cc.	24	No symptoms
106	3	7 days	Early	6.0	RT, 1.0 cc.	24	No symptoms
111	3	7 days	Early	6.0	RT, 1.0 cc.	24	No symptoms
316	1	Months	Early	6.0	RT, 1.0 cc.	24	No symptoms

\* The R1 extract was injected ten minutes after the RT injection.

The failure of the passive transfer of the pollen hypersensitive-ness from hay fever subjects to guinea pigs may have been due to one of two causes: either it was due to the proven insus-ceptibility of the guinea pigs to sensitization with pollen sub-stances, or it was due to the absence of antibodies to those substances in the blood of the hay fever patients.

It is possible to throw light on this question by carrying out similar experiments with the blood of individuals naturally sen-

sitized to substances to which guinea pigs may be artificially sensitized. This we have done, injecting subcutaneously into guinea pigs 6 cc. of the active citrate plasma of two individuals that were hypersensitive to horse epithelium. Four of these animals received on the following day, intravenously, more than two times the minimal lethal dose of an extract of horse epithelium. None of the guinea pigs so treated showed any symptom of anaphylactic shock (tables 3 and 4). *These consistent results indicate the absence of antibodies in the blood of naturally hypersensitive human individuals.*

TABLE 3

*Preliminary tests to determine the minimal toxic dose of the extract of horse epithelium (curryings)*

GUINEA PIG	JUNE 6 S. C. INJECTION	JUNE 20 I. V. INJECTION	RESULTS
237	H. Ep. Ext., 0.5 cc.	H. Ep. Ext., 0.5 cc.	Typical anaphylactic death
227	H. Ep. Ext., 0.5 cc.	H. Ep. Ext., 0.1 cc.	Typical anaphylactic death
245	H. Ep. Ext., 0.5 cc.	H. Ep. Ext., 0.1 cc.	Slight anaphylactic symptoms recovered.
241	H. Ep. Ext., 0.5 cc.	H. Ep. Ext., 0.05 cc.	Slight anaphylactic symptoms recovered
244	H. Ep. Ext., 0.5 cc.	H. S., 0.15	No symptoms
238	H. S., 0.025 cc.	H. S., 0.15 cc.	Typical anaphylactic death
240	H. S., 0.025 cc.	H. Ep. Ext., 0.5 cc.	No symptoms

s.c. = subcutaneous. i.v. = intravenous. H.Ep.Ext. = Extract of horse curryings. H.S. = Horse serum.

Assembling the available facts, clinical and experimental, relating to hay fever, we may formulate the following conception of that form, and indeed of all forms, of natural human sensitization:

(1) Hay fever is the clinical symptomatic expression of local hypersensitiveness. The active pollen substances are not toxins.

(2) *The hypersensitiveness is established spontaneously and never by immunological process.* This has been shown in two ways; first by the observation of Dunbar (13), confirmed by Cooke, that individuals may be sensitive to pollens of plants that are indigenous in foreign countries and with which they have never



come in contact; and secondly, by the observation of Cooke that individuals who are naturally sensitive to one protein only can not be artificially sensitized to another protein, either animal or vegetable.

(3) *The sensitization is not directly inherited (Cooke and Vander Veer), although the tendency to spontaneous sensitization is inherited as a dominant character.*

(4) The antibody-like substances of human sensitization are not demonstrable in the blood of sensitive persons by any of the

TABLE 4

*Showing absence of sensitizing antibodies to horse epithelium in the serum of human individuals naturally hypersensitive to horse epithelium*

GUINEA PIG	JUNE 30 S. C. INJECTION	JULY 1 I. V. INJECTION	RESULTS
247	Serum (active) 6.0 cc. E. F. S. (614)	H. Ep. Ext., 0.4 cc.	No symptoms
248	E. F. S. (614)	H. Ep. Ext., 0.4 cc.	No symptoms
251	Serum (active) 6.0 cc. G. J. W. (479)	H. Ep. Ext., 0.4 cc.	No symptoms
253	G. J. W. (479)	H. Ep. Ext., 0.4 cc.	No symptoms

E. F. S. Had had 17 injections, from 0.2 to 1.0 cc., of horse epithelium extract, the last injection having been given on June 15. On January 29 the patient was clinically entirely free from symptoms and the ophthalmic reaction was negative.

G. J. W. Beginning March 4 of the same year to June 29 had had 11 injections, the maximum amount injected at one time being 0.05 cc. He was free from symptoms on June 29.

immunity reactions. They are present in the cells of the sensitive tissues. They can not be increased artificially by the usual process of immunization.

(5) The mechanism of the alleviating effect of specific, i.e., vaccine therapy, is the same as that of desensitization in experimental anaphylaxis. The freedom from symptoms lasts as long as the respective "antigenic" substances remain in combination with the antibody-like substances in the tissues.

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# IMMUNOCHEMICAL STUDIES OF THE PROTEINS OF CAT HAIR

## STUDY VI<sup>1</sup>

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It had been shown by Dr. J. L. Goodale ('16) and others that cats were frequently the cause of asthma and that if some of the hair from these animals be soaked in 12 per cent alcohol an extract is obtained which can be used to produce the typical skin reaction now so much used in the diagnosis of asthma and hay-fever (cf. Goodale, '16, Talbot, '16, Walker, IV, this series).

Hitherto cat asthmatics have never been treated with cat hair extracts, as has been done in other types of asthma and in hay-fever, to produce immunity, on account of the difficulty of obtaining an extract from the hair suitable for making hypodermic injections. Since it is so easy with 15 per cent alcohol

<sup>1</sup> This is the sixth of a series of papers on the study of bronchial asthma made possible through a gift by Mr. Charles F. Choate, Jr., Boston, to the Peter Bent Brigham Hospital. The papers previously published are:

Study I, Studies with the staphylococci pyogenes, aureus, albus and citreus and with the micrococci tetragenus and catarrhalis. I. C. Walker and June Adkinson, *Journ. Med. Res.*, 1917; **35**, 3; 373.

Study II, Studies with a diphtheroid organism isolated from the sputum of patients with bronchial asthma. Walker and Adkinson, *Journ. Med. Res.*, 1917; **35**, 3; 391.

Study III, Studies on the sensitization of patients with bronchial asthma to bacterial proteins as demonstrated by the skin reaction and the method employed in the preparation of these proteins, I. C. Walker, *Journ. Med. Res.*, 1917; **35**, 3; 487.

Study IV, Studies on the sensitization of patients with bronchial asthma to the different proteins found in the dander of horse and the hair of the cat and of the dog and to the sera of these animals. I. C. Walker, *Journ. Med. Res.*, 1917; **35**, 3; 497.

Study V, Studies on the sensitization of patients with bronchial asthma to the different proteins in wheat and to the whole protein of wheat, corn, rice, barley, rye and oat. I. C. Walker, *Journ. Med. Res.*, 1917; **35**, 3; 509.

to make an extract from cat hair that is strongly anaphylactogenic it seemed certain that the hair must contain in it or on it some substance that is more soluble than the extremely resistant keratin of which it is mainly composed. It also seemed improbable that keratin could be the substance in the hair causing "hay-fever." This investigation, therefore, was undertaken to find out the nature of the other substance in the hair and, if possible, to establish some simple method of preparing it in a form convenient for diagnosis and treatment.

Thirty grams of hair were procured by clipping a number of cats in the laboratory. No attempt was made to clean the hair because some experiments of Dr. I. C. Walker had shown that, in the case of dogs, the more unkempt the animals were the more active was a 14 per cent alcoholic extract; it seemed probable, therefore, that washing would remove the very substance that gave the hair its anaphylactogenic properties. However, only clean looking hair was used, pains being taken to see that it was free from visible foreign matter. The hair was extracted in 1 liter of 0.2 per cent KOH at about 65° for three or four hours. This was repeated three times, the mixed extracts producing a clear yellow solution. This solution was neutralized with weak acetic acid, which caused the formation of a yellow-brown precipitate reaching a maximum somewhat to the acid side of neutrality to litmus. This precipitate was filtered out and washed with water on the filter but it was found to be partly soluble in water. This was corrected, however, by making the water slightly acid to litmus. In order to purify it further the precipitate was dissolved on the filter in 0.2 per cent KOH, in which it proved to be almost completely soluble, leaving on the filter only a very small amount of dark "insoluble residue," which was kept for further investigation. This solution was again neutralized with acetic acid, causing the reappearance of the brown precipitate, which was centrifugalized out and washed through two baths each of water (slightly acidulated), 95 per cent alcohol and ether. When the precipitate was dried in the desiccator under diminished pressure, a dark brown powder was obtained of which there was 0.05 gram from the original

30 grams of hair (i.e., 0.15 per cent of the hair) which means that one cat yields 0.025 gram or 1 gram is the yield of forty cats.

This powder is completely soluble in N/100 KOH giving a dark brown solution, and when so dissolved it can be precipitated by adding acetic acid until faintly acid but it redissolves upon further addition of the acid. It gives all of the protein color tests. It does not, however, give the Molisch reaction, thus showing that it is a protein without a carbohydrate group. According to the nitrogen determination made on this preparation by Miss G. Russell of this hospital and, assuming that what protein there is in it contains 16 per cent of nitrogen, which is the average for ordinary proteins, the preparation was shown to be composed of 60 per cent protein. It was thought from the solubility characteristics of this protein that it was probably a phosphoprotein or a nucleoprotein. Accordingly it was tested with this in view but neither phosphorous nor purine bases could be detected. However the difficulty of making satisfactory tests on the small amount available leaves this still uncertain, therefore it is provisionally called "alkali metaprotein."

In order to find out whether this is the suspected anaphylactogenic protein of cat hair it was tested on a number of patients. In the few cases tried at this stage of the work it was found that wherever the 14 per cent alcoholic extract gave a reaction the protein always gave one as strong and, in some cases, very much stronger.

An attempt was made to purify this alkali metaprotein still further. It was dissolved in N/100 KOH. There was always a small part, however, that would not dissolve. This was centrifugalized out of solution and dried in the form of a white powder. The amount of this substance obtained was too small to allow satisfactory tests. However, it did not seem to possess any protein characteristics and when tried on sensitive patients with the skin test it failed to give any reaction. This fraction, therefore, is called the "Insoluble residue." The "Extra pure metaprotein" which was precipitated from this solution and dried in the usual way differed in no way from the original alkali metaprotein.

Many experiments were tried to find some method of increasing the yield but in no way could a greater proportion of protein be obtained. On the contrary, whenever the process described above was deviated from and even sometimes when it was followed as exactly as possible, a much smaller amount or sometimes none at all was obtained. On one occasion, when the method above was followed as nearly as possible, after the precipitate was formed and redissolved in 0.2 per cent KOH it could not be caused to precipitate again by neutralization with HCl nor even by making acid; it was therefore made alkaline and again neutralized and acidified but no precipitate could be caused to form. From this it seemed that the protein had been changed in some way to a much more soluble form. Consequently saturation with ammonium sulphate was resorted to and this caused an almost complete precipitation of the protein. This precipitate was then centrifugalized out and found to be insoluble in water; it was therefore washed in water to remove the sulphate and dried in alcohol and ether, desiccation being completed in a desiccator producing only a small amount of black powder. This is undeniably in substance the same protein as the alkali metaprotein described above although it has very different properties owing to its different method of preparation. For convenience it is called "Cat hair protein no 2." This was tried on only one patient but it gave a scarcely appreciable reaction although the patient at the same time gave a very strong reaction to the alkali metaprotein; consequently this method of preparation was abandoned.

On account of the extremely small amount of active protein obtained by neutralization of the alkaline extract and because this amount, even small as it was, could not be depended upon, the filtrate was investigated. It was greatly reduced in volume by boiling and a few chemical tests showed that it contained a large amount of protein giving all the protein color reactions and the precipitation reactions which would indicate a peptone together with traces of another substance. It was also tested on a number of patients and found to give approximately the same skin reactions as the alkali metaprotein. From this it was in-

ferred that this method of extraction with alkali and precipitation with HCl had the effect of hydrolysing some of the active protein of the hair, for the greater part, into peptone that only a small amount of it was obtained in the form of the alkali metaprotein; it was thought, therefore, that bivalent or trivalent salts might have the effect of throwing it out of solution *in toto*. A number of these salts were tried on parts of a freshly prepared alkaline extract which had been approximately neutralized without producing an appreciable precipitate, and it was found that magnesium chloride would bring about almost complete precipitation of the protein even when used in low concentration (viz., 0.008 M). The precipitate thus obtained when dried in the usual way had the same appearance as the alkali metaprotein but proved to be totally insoluble in weak alkali, neutral or acid solutions, either hot or cold. The amount obtained was too small to be characterized further, therefore it is called, for the time being, "Cat hair protein no 3." It was tried out with the skin test and found to give only a -1 reaction on a patient, who, at the same time, gave a reaction rated at 4 (according to Goodale's nomenclature) to the alkali metaprotein. This method of precipitating the protein, therefore, was abandoned.

The filtrate left after the alkali metaprotein had been precipitated with HCl was made faintly alkaline by means of KOH, whereupon a fair amount of protein was precipitated. This was found to be insoluble in neutral solution but it dissolved in faintly acid solution forming an opalescent fluid. It was centrifugalized and washed several times in faintly alkaline water then in two baths each of 95 per cent alcohol, absolute alcohol and ether, and when dried it formed a small amount of pure white powder. Since this substance is soluble in acid and insoluble in alkali, always forming a gelatinous precipitate with the latter, it is called "acid metaprotein." The amount of the "acid metaprotein" obtained was too small to make any satisfactory tests, but it showed the xanthoproteic and Molisch reactions, indicating that it is a protein containing a carbohydrate group. So far it is characterized solely by being soluble in acid and insoluble in alkali. In no case did it give an appreciable

skin reaction. The filtrate from this, that is to say the filtrate from the alkali metaprotein reduced in volume by boiling, neutralized and having had the acid metaprotein removed, was dialysed to free it from Cl and, since this was found not to impair its anaphylactogenic properties, it was then dialysed first against 95 per cent alcohol, then against absolute alcohol until all the water had been replaced by alcohol. This caused the production of a brown precipitate, which, after being washed in absolute alcohol and then with ether was dried over sulphuric acid under diminished pressure. The result was the production of a small amount of gray powder which is called "cat hair peptone" on account of its characteristics. It is extremely soluble in water even taking up water from the atmosphere. It also gives the characteristic precipitation reactions of peptone. Nitrogen determinations were made on this by Miss Russell by the Kjeldahl method and, assuming that this protein has the average amount of nitrogen in it "Cat hair peptone" was shown to be 50 per cent pure protein. A glance at the table will show that this is the most active protein obtained from cat hair.

#### PURE KERATIN

Keratin is the most insoluble of all the animal proteins. It is the substance which makes up the principal part of all kinds of horn, hair, nails, hoofs, etc., which are necessarily very resistant. It is considered a schleroprotein or albumoid, somewhat related to collagen and elastin, the principal proteins of connective tissue; also to spongin, the principle substance of the skeletal tissue of the domestic sponge. The schleroproteins as a group are all very insoluble bodies. Keratin, however, can be distinguished by its resistance to enzymes and its high content of sulphur.

This characteristic resistance to solvents and digestive ferments is the one made use of in the purification of keratin. The hair was washed in hot water until all soluble contaminations were removed and the water came away clear. It was then boiled in 1 per cent HCl for several hours, thoroughly washed in hot water and then digested with acid pepsin for two days, which



seemed to dissolve a small part of the hair, causing it to disintegrate. After the residue was thoroughly washed it was digested in alkali-pancreatin for two days, then washed and digested again in acid-pepsin. After this it was boiled again in 1 per cent HCl, boiled in water and thoroughly washed, boiled in absolute alcohol, washed in water and dried over sulphuric acid. When dried a gray powder was obtained and this was put through the whole process again. The keratin so prepared had completely lost the original structure of the hair, having the appearance of a light gray flaky powder. Some of this was soaked in as small an amount of 14 per cent alcohol as possible and skin tests were made with the solution; but in no case so far has a positive reaction been obtained.

#### KERATIN DECOMPOSITION

It was thought possible that the hair proteins described above might be simply decomposition products of keratin formed by a natural hydrolysis or through the action of the reagents used in their preparation. In order to test this, some of the keratin, which had been shown by the skin tests to be inactive, was hydrolyzed by means of the ordinary method with hydrochloric acid. It was boiled in 5 per cent HCl for three days, or until most of the keratin was dissolved and the filtrate gave a deep reddish purple biuret reaction. The resulting mixture was then centrifugalized and the clear solution obtained was dialysed free from Cl and evaporated to dryness, when a fair amount of gray powder was obtained which is perfectly soluble in water and has all the characteristics of peptone. This substance is called "cat keratin peptone." That it is not the same as any of the other proteins obtained from the hair is shown by the different skin reactions that it gives, as will be seen by a glance at the table below.

The cat serum preparation used in this experiment was made by Dr. I. C. Walker by precipitating all the proteins of normal serum by adding three or four volumes of acetone and washing the precipitate in alcohol and ether and drying in the desiccator.

This method of treatment produced a white powder soluble in water or dilute alkali.

In the following table + indicates a definite reaction while = indicates that the reaction was so slight that it could be regarded as merely suspicious; ++ indicates that the reaction was very large; and in most cases when such a reaction was obtained the protein was dissolved in N/100 KOH and diluted in the following geometric series, 1: 100, 1: 1000, 1: 10,000, 1: 100,000, 1:1,000,000 and these dilutions were tried by means of the skin test to see to what extent the protein could be diluted and still give the reaction. When this was done the greatest dilution with which a reaction was obtained was recorded in the table together with the value of the reaction produced. Many of these tests were repeated and, though there was a small amount of variation, there is every indication that all these reactions could have been repeated at the same time with the same result. If the tests were repeated after a long interval there was often considerable variation (Walker, Study IV, this series).

*Cat hair proteins*

PATIENT	HAIR 14 PER CENT ALCOHOL EXTRACT	SERUM	PURE KERATIN	KERATIN PEPTONE	ACID METAPROTEIN	ALKALI METAPROTEIN	PEPTONE
C. N. E.....	+	+	0	±	0	1: 10,000 ±	1: 100 0
D. A. G.....	+	0	0	0		1: 1,000 ±	1: 1,000 ±
C. J.....	+	+	0	+	0	1: 1,000 +	1: 100 ±
F. A.....	+	+	0	0		1: 100 +	1: 100 +
P. D.....	+	+	0	±		1: 1,000 ±	1: 10,000 ±
J. H. N.....	+	+			0	1: 10,000 ±	1: 1,000 ±
S. H. G.....	+	0	?	++	++	1: 1,000 +	1: 100 +
G. V. Y.....	+	0	0	0	±	1: 1,000,000 +	1: 1,000,000 +
M. D.....	+					1: 10,000 +	1: 10,000 +

SUMMARY AND CONCLUSIONS

Besides "keratin," which makes up the greater part of the hair of most animals, there can be obtained from cat hair other proteins and these differ both in chemical characteristics and ana-

phylactogenic behavior from pure keratin and from a peptone which was produced by acid hydrolysis from pure keratin and which is called in these experiments "keratin peptone."

Other proteins obtained from cat hair were as follows:

"Alkali metaprotein," the protein dissolved from hair by weak alkali which is precipitated by neutralizing and faintly acidulating.

"Acid metaprotein," the protein that is dissolved from hair by weak alkali and remains in solution after neutralizing and slightly acidulating but is precipitated by again making alkaline.

"Cat hair peptone," the protein dissolved from the hair by weak alkali that remains in solution after the other two are precipitated as indicated above. It is obtained by evaporating the solution to dryness after removing the mineral salts by dialysis.

If these proteins dissolved from the hair by weak alkali are precipitated by other means their anaphylactogenic properties may be altered. For example:

"Protein preparation No. 2," prepared by saturating the alkaline extract with ammonium sulphate; and

"Protein preparation No. 3," precipitated from an alkaline extract (from which no protein could be obtained by neutralization or acidulation) by the addition of a small amount of  $MgCl_2$  were comparatively inactive.

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# IMMUNOCHEMISTRY OF THE PROTEINS OF HORSE DANDER

## STUDY VII<sup>1</sup>

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The material used in these experiments was groomings from horses procured by Dr. I. C. Walker and consisted principally of epidermal desquamations with a small proportion of hair. Some of the hair was sifted out and used for the preparation of pure keratin. The rest of the material was put into N/100 KOH and kept at about 80°C. for about one hour in the water bath. This mixture which had the appearance of thin mud mixed with a little hair, was centrifugalized and the supernatant fluid (which was not quite clear) was further cleared by filtration, and neutralized with 1 per cent HCl. As the acid was added a large amount of a light gray precipitate was formed, reaching a maximum a little to the acid side of neutrality to litmus. The precipitate, which was very fine and consequently separated only with difficulty, was centrifugalized out and washed through two baths of water and redissolved in N/100 KOH, in which it was almost completely soluble, giving a clear yellow fluid. This was precipitated again by neutralization with HCl and the precipitate washed through two baths each of water, 95 per cent alcohol, absolute alcohol and ether and dried over sulphuric acid with diminished pressure. When desiccation was complete a little over 1 gram of a light brown powder was obtained, which was 2.47 per cent of the original material.

This protein is called "alkali metaprotein" on account of its properties. It is soluble in weak alkali and precipitated by neutralization, but it is soluble in a small excess of acetic acid.

<sup>1</sup> See Study VI, this journal, p. 227.

It is insoluble in 10 per cent NaCl. Chemical tests showed the absence of purine bases and of phosphorous, thus showing that it is not a nucleoprotein nor phospho-protein. It gives all of the protein color reactions very decidedly but no Molisch, showing the absence of a carbohydrate group. A glance at the table will show that this is one of the most anaphylactically active proteins obtained, always giving as large a reaction as does the 14 per cent alcoholic extract of the hair, and generally a much larger one.

It was observed that the alcohol baths dissolved a large amount of the protein from the above preparation. The alcoholic extracts were, therefore, mixed and evaporated to dryness. This produced a white substance which was scraped off the evaporating dish and ground to a powder. The powder is only sparingly soluble in water, weak alkali or alcohol, but it is soluble in strong alkali (viz., N/10 KOH); in most cases it is anaphylactically very inactive. But that this is not due to any constitutional peculiarity of the protein is shown by the case of L. B. C., with whom it is as active as any other of the other preparations.

The filtrate from the alkali metaprotein, which was precipitated by neutralization with HCl, was reduced in volume by evaporation. During the process, the first part of which was carried out by boiling on a wire gauze and was long continued on account of the large volume, a coagulum was produced by the heat. Boiling was discontinued before evaporation was complete and the coagulum was filtered out, washed in water and redissolved in N/1000 KOH, in which it was found to be almost completely soluble, producing a clear yellow solution. Though this substance was quite insoluble in pure water and could only be dissolved by dilute alkali, having once been dissolved, it could not again be precipitated by neutralization or even by acidification, showing that solution by alkali caused some structural change. This solution was then dialysed free of Cl and evaporated to dryness on the hot water bath producing a white powder. It is called provisionally "horse hair coagulated protein."

The filtrate from this heat "coagulated protein" was dialysed

(without further reduction in volume) free from Cl, then dialysed into alcohol, which reduced the volume to about one-third and caused a small amount of precipitate. Precipitation was completed by adding several volumes of acetone and ether. When the precipitate was dried in the usual way, a white powder was obtained weighing only a fraction of a gram. This substance is readily soluble in either pure water or weak alkali and has the characteristics of a peptone. It gives a precipitate with phosphotungstic, tannic and picric acids but not with trichloroacetic; it is, therefore, called provisionally "horse hair peptone." The skin tests (see table) show this to be generally as active anaphylactically as the alkali metaprotein, but that this is not the same as the latter substance is shown by such cases as F. A., J. M., D. G., or R. H. W., where it gives no reaction, while both the alkali meta- and coagulated proteins do.

#### PURE KERATIN

The hair from some of the horse groomings was shaken free from the desquamations that accompanied it and washed in hot water until all soluble contaminations were removed and the washings came clear. It was then boiled in 1 per cent HCl and again washed in hot water. When the water would remove no further impurities the hair was digested with acid pepsin for two days, which seemed to dissolve a small proportion of the hairs and cause them to disintegrate. After being thoroughly washed the residue was digested in alkali pancreatin for two days. It was then again digested with acid pepsin. After this it was boiled again with 1 per cent HCl, boiled in water and thoroughly washed, boiled in absolute alcohol, ether and dried over sulphuric acid.

After this treatment it had almost entirely lost the structure of the individual hairs so that macroscopically the whole mass was a very light and fluffy brown powder. Microscopically, however, it was seen to consist, for the most part, of micellar bodies perfectly uniform both as to size and form.

Skin tests were made on a large number of asthmatics, who

readily responded to the 14 per cent alcoholic extract of the hair, but in no case was an indisputable reaction obtained. This shows that it is not the keratin of the horse hair which is responsible for its anaphylactogenic properties.

#### KERATIN SPLIT PRODUCTS

Since the keratin is not anaphylactogenic it seemed possible that this property of the hair might be due to the decomposition products of the keratin and that the highly active proteins described above might owe their origin to a natural decomposition of the keratin or to its hydrolysis during extraction and purification. Consequently, some of the keratin prepared as described above, was split up by the ordinary method of acid hydrolysis (viz., boiling for about three days in 5 per cent HCl). Most of the keratin was dissolved producing a clear brown fluid which was filtered off and shown by chemical tests to contain a large amount of peptone. This solution was dialysed free from Cl and evaporated upon the hot water bath. A large amount of brown powder was produced, which was found to be soluble in water and to give all the peptone tests.

This "keratin peptone," as it is called, was tried by means of the skin test upon a number of highly sensitized horse asthmatics but, as a glance at the table will show, in no case did it give more than a doubtful reaction. Hence the decomposition products obtained by this method from keratin are not responsible for the anaphylactic reaction of the hair.

In the following table is shown a comparison of the reactions given by patients tested with the different proteins of horse dander and with the serum. The tests were all made by Dr. I. C. Walker and Miss June Adkinson as explained by Walker in the fourth paper of this series, and to them am I indebted for the privilege of using these records. The method employed was to make a series of abrasions on the inner side of the forearm of patients suspected of being horse-sensitive and upon each of these was placed a small amount of the protein which was then moistened with a drop of N/100 KOH. In recording the results



+ indicates a decided reaction; = indicates an extremely doubtful reaction, while ++ indicates a very strong reaction. In practically all cases, however, where a ++ reaction was obtained the protein was dissolved in N/100 KOH in the proportions of 1:100, 1:1000, etc., and the series of dilutions thus obtained were tried on a series of abrasions. The greatest dilution to give a reaction is the one recorded in the table.

Some of the patients appearing in this table were also sensitive to the proteins of cat hair. They may be recognized by their initials (c.f. Study IV, this journal).

PATIENT	HAIR	SERUM	PURE KERATIN	PEPTONE	ALCOHOL SOLUBLE PROTEIN	ALKALI METAPROTEIN	PEPTONE	COAGULATED PROTEIN			
D. A. G.....	+	≠	0	0	0	1: 10,000	+	1: 10,000	+		
C. N. E.....	+	+	0	0	+	1: 10,000	+	1: 10,000	+		
C. J.....	+	0	0	0	0	1: 1,000	+	1: 10,000	+		
F. A.....	+	0	0	0	0	1: 10,000	≠	0	1: 10,000	≠	
H. M.....	+	+	0	0			1: 100	≠		≠	
L. T.....	+	0	0	0		1: 10,000	+	1: 10,000	+	1: 100,000	+
J. M.....	+	≠	0	0		1: 100,000	+	0	1: 1,000	+	
C. M.....	+	0	0	≠	≠	1: 1,000	≠	1: 1,000	≠	1: 1,000	≠
D. G.....	+	0	0	0	≠	1: 100,000	≠		≠	1: 100	≠
R. H. W.....	+	0			0	1: 10,000	≠	0	1: 10,000	≠	
P. D.....	+	+	0	≠		1: 1,000	≠	1: 1,000	≠	1: 100	≠
G. B.....	+	0	0	0		1: 10,000	≠	1: 10,000	0	1: 10,000	≠
S. H. G.....	+	0	0	0	0	1: 10,000	≠	1: 10,000	+	1: 10,000	≠
L. B. C.....	+	0	0	0	1.100	+	+	1: 100,000	+	1: 100,000	+
C.....	+	0	0	0	0	1: 10,000	≠	1: 10,000	+	1: 10,000	≠

From the table it can be seen that the different proteins are immunologically distinct. Their chemical reactions also show them to be chemically different. However the method of preparation and the fact that none has as yet been crystallized precludes the possibility of obtaining them in an absolutely pure form.

#### SUMMARY AND CONCLUSIONS

From horse dander there can be dissolved out with weak alkali a large amount of protein matter. Part of this can be

precipitated by neutralization with HCl, and if this is treated with 95 per cent alcohol the major portion, the "*alkali meta-protein*," remains undissolved, while a very much smaller portion, the "*alcohol soluble protein*," is dissolved by the alcohol. Of the protein substance that is not precipitated by neutralization by HCl a portion, the "*heat coagulated protein*," can be precipitated by boiling, when the reaction is slightly acid. After this is removed there still remains in the solution another protein, the "*hair peptone*."

Pure keratin was also prepared from the hair and from this a peptone, "*keratin peptone*," was prepared by hydrolysing with HCl.

When tested by means of the skin reaction the four proteins dissolved out from the dander by dilute alkali showed distinctly different anaphylactogenic properties and they are likewise different from the serum.

Pure keratin is inactive towards horse asthmatics; so, also, is a peptone prepared by acid hydrolysis from it.

From this it is concluded that there are, besides keratin, at least four proteins present in horse dander and that these are not decomposition products of keratin.

# IMMUNOCHEMISTRY OF THE PROTEINS OF DOG HAIR

## STUDY VIII<sup>1</sup>

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A large amount of hair was obtained partly by clipping dogs in the laboratory and partly from a professional dog clipper. No attempt was made to clean the hair in any way but only clean looking hair was used. The whole mass was soaked in N/100 KOH. After being allowed to soak over night it was squeezed out and the liquid filtered off producing a clear yellow solution. Two more extracts were made from the same hair and all three were mixed and neutralized with HCl. A large amount of brown precipitate was thrown down, the production reaching a maximum somewhat to the acid side of neutrality to litmus. Great care had to be taken not to pass the optimum acidity for if this had been done the precipitate would have dissolved again in the acid and neutralization again would have caused the reappearance of only a small amount of it.

The precipitate was next filtered out, washed several times in water and redissolved in N/100 KOH, in which it is completely soluble. It was again precipitated with HCl and centrifugalized out and washed through two baths each of water, 95 per cent alcohol, absolute alcohol and ether and dried over sulphuric acid under diminished pressure. About 2 grams of a black powder were obtained. This substance gives all of the protein color tests and a positive Molisch reaction. It is soluble in weak alkali or weak acid but is insoluble in neutral solutions and can be precipitated from solution by neutralizing and then making

<sup>1</sup> See Study VI, this journal, p. 227.

slightly acid but it is redissolved upon further acidification. It can, however, again be precipitated by reducing the acidity to near neutrality and redissolved by making alkaline; then it can be precipitated again by HCl but this precipitate is permanent and will not dissolve in either weak acid or weak alkali.

Since the solubility characteristics of this substance resemble those of phosphoprotein and nucleoprotein it was examined for phosphorous and purine bases. However, as no phosphorous nor purine could be demonstrated in it this protein is called provisionally "Dog hair alkali metaprotein."

The filtrate from which this protein had been precipitated by HCl, having been found to contain protein, was reduced from a volume of about 8 litres to about 100 cc. It was then dialysed free from Cl, then dialysed into 95 per cent alcohol, which still further reduced the volume and precipitated part of the protein. Precipitation was completed by adding three volumes of a mixture of acetone and ether (4 to 1). The precipitate was centrifugalized out and dried in acetone and ether, desiccation being completed over sulphuric acid under diminished pressure.

This protein, of which less than one gram was obtained from a kilo of hair, is a red-brown powder readily soluble in water and deliquescent in the atmosphere. Since it has the characteristics of peptone it is called "dog hair peptone."

#### PURE KERATIN

Pure keratin was obtained from dog hair in almost exactly the same way as from horse hair (Study VII). In this case, however, the keratin retained almost entirely the structure and colour of the hair from which it was prepared.

#### KERATIN DECOMPOSITION

In order to see whether the decomposition products of keratin could be responsible for the anaphylactogenic properties of the hair some purified keratin which had been shown by the skin test to be free from anaphylactogenic properties, was hydrolyzed in the ordinary way by boiling in 5 per cent HCl for three days.

The filtrate was then dialysed free from Cl and evaporated to dryness. A small amount of light gray powder was obtained, which, since it was found to be very soluble in water and to have the characteristics of a peptone, is called "dog hair keratin peptone."

Tests were made with these proteins by Dr. I. C. Walker and Miss June Adkinson as described by Walker (Study IV of this series. See also Studies VI and VII, this journal), and to them I am indebted for the use of the accompanying table which shows the reactions obtained with these proteins on seven different patients asthmatic to dogs.

PATIENT	HAIR, 14 PER CENT ALCO- HOL EX- TRACT	SERUM	PURE KERA- TIN	KERA- TIN PEP- TONE	ALKALI METAPROTEIN	PEPTONE
D. A. G.....	+	0	0	0	1: 100 ±	1: 100 ±
C. N. E.....	+	+	0	±	1: 100 ±	1: 10,000 +
C. J.....	+	0	0	+	1: 10,000 ±	1: 100 0
F. A.....	+	+	0	0	1: 100 +	1: 100 0
H. M.....	+	0	0	0	1: 1000 ±	1: 1000 ±
P. D.....	+	+	0	±	1: 1000 ±	1: 100 0
L. B. C.....	+	0	0	0	1: 100 ±	1: 100 +

In the above table ± indicates a very faint reaction which possibly should, in some cases, be regarded as negative; + indicates a perfectly definite reaction but nevertheless so small that further dilution of the protein would reduce it to zero.

The tests were made by applying the protein in powder form to small abrasions in the skin of the forearm and moistening with N/100 KOH. Since the sizes of the reactions obtained give a very inadequate comparison of the anaphylactogenic activities of the proteins, those which generally gave larger reactions than that indicated by a + were dissolved in N/100 KOH in the series of concentrations 1: 100, 1: 1000, etc., and these solutions were used in making the tests instead of the pure proteins.

In the tables are also included the results of tests made with serum protein because it was thought possible that there was some immunological connection between the hair of an animal and its blood serum.

From these experiments it can be seen that the proteins of the hair bear but little anaphylactogenic relation to those of the serum and that the two proteins obtained from the hair by extraction with weak alkali bear little or no relation to the peptone prepared by acid hydrolysis of the pure keratin. There is also some evidence that the alkali metaprotein is distinct from the peptone, though both are anaphylactogenic; for in the case of C. N. E. the peptone is over one hundred times as active as the alkali metaprotein, while in the case of C. J. the alkali metaprotein is at least a thousand times as active as the peptone.

#### SUMMARY AND CONCLUSIONS

From dog hair there can be dissolved out with weak alkali a small amount of protein. Part of this, the "*alkali metaprotein*," can be precipitated by neutralization while the rest remains dissolved but can be recovered from solution by dialysing out the salt and evaporating to dryness. This we have called "*dog hair peptone*."

These two proteins show themselves, by their skin reactions with asthmatics, to be immunologically distinct and also distinct from a peptone obtained from pure keratin, which is itself inactive, by hydrolysis, and from the serum proteins of the same species.

# ON HYPERLEUCOCYTOSIS AND ITS BEARING ON SPECIFIC THERAPY

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The literature of the last ten years contains a great many reports of the successful treatment of various acute infectious diseases with suspensions of the dead bacteria causing the disease, i.e., homologous "vaccines." However, although this method has attained universal acceptance as a *prophylactic* measure in typhoid fever, cholera and paratyphoid infections, and promises similar prospects in some other conditions, it can hardly be said that it has been more than hopefully suggestive as a therapeutic procedure used during the course of actual disease. Indeed there has been an increasing suspicion among bacteriologists that the effects produced, when bacterial protein was injected into patients, were not in any sense specific, and depended rather upon the general character of the injected substances as bacterial proteins, than upon their specifically immunizing properties.

Jobling and Peterson have recently summarized this point of view in an interesting paper in which they have cited much of the pertinent literature; and have again called attention to the fact that attempts at specific bacterial vaccine therapy in acute disease, are by no means a very recent development, but were made by Frankel in typhoid fever as early as 1893.

In this malady particularly, efforts with such methods have been numerous and persistent, and favorable reports have come from Petrushky (1902), Netter and others. Ichikawa in 1912, and Boinet in 1914 obtained astonishing results by using sensitized vaccines, the former introducing the intravenous method,

and the same procedure has been more lately followed by Gay. There can be little doubt, at least for typhoid fever, that the administration of typhoid vaccine often results in striking effects in the patients—in the form of chills and drop in temperature, and—objectively, Boinet claims a rapid mobilization of bactericidal antibodies. Whether, these changes are specific or not, however, is by no means certain, indeed it is very much to be questioned. Kraus, following the work of Ichikawa, obtained results not unlike those of the latter by injecting colon bacilli into typhoid patients and, with the same vaccines, succeeded in exerting similar effects upon a few cases of pyocyaneus infection and streptococcus puerperal septicaemia.

We have been interested in non-specific therapy of this nature for many years, indeed since 1906, when one of the writers was associated with Prof. Hiss in the study of the therapeutic action of leucocytic extracts. Although at the time this work has done it was believed most likely that the extracts possessed poison-neutralizing properties, we soon afterward formed the opinion that the mobilization of leucocytes, as a consequence of the injection of the extracts, was the true reason for their occasional beneficial action. This view was based largely on white cell counts made in this laboratory by Miss Gilbert, and on the protective influence of the extracts upon plague rats, observed with McCoy and Chapin, since as far as we know no poisons can be extracted or otherwise obtained from these bacilli.

We were led for these reasons to assume that the action of the leucocytic extracts was similar to that of the nucleic acid solutions as used by Miculiez, to the so-called "phylacogens" of Schaeffer and various other non-specific agents similarly used by others and more fully discussed in the paper of Jobling and Peterson. These writers also considered that the action of the various non-specific agents might be due in part to leucocyte mobilization but add the suggestion that hyperpyrexia and the rapid appearance of increased amounts of serum protease and lipase, may also play an important rôle.

Granted then that the course of an acute infectious disease may be non-specifically altered by the intravenous injection of



heterologous bacteria and other protein substances the important question arises whether or not these forms of therapeutic effort are for any reason inferior to the use of homologous bacteria, i.e., specific vaccines.

Bordet, as early as 1896 made the following statement, "Active immunity has also other characteristics, in that there is an increase of the number of leucocytes, that is, an "exaltation" of the chemotactic sensibilities of the leucocytes." He suggests herein that an immunized animal may respond with a more powerful leucocytic reaction to the injection of the infectious agent than would a normal animal similarly treated. This idea has recently found experimental elaboration in the work of Gay and Claypole, who found that the reinjection of immune animals with the homologous bacteria produced a specific hyperleucocytosis, that is, typhoid immune animals receiving typhoid bacilli would respond with counts ranging up to 150,000 leucocytes per cu. mm., whereas the normal animals rarely showed more than 40,000 to 50,000. Furthermore the injection of other organisms such as *micrococcus aureus* into typhoid immune animals produced reactions entirely parallel to those observed in similarly treated normal rabbits.

This observation would tend to indicate a great advantage of the specific over the non-specific methods of treatment and would offer a most reasonable explanation for the success of specific therapy in general.

Unfortunately the results of Gay and Claypole have not found confirmation. McWilliams in similar experiments found no differences in the degree of leucocytosis between normal and immune animals in response to the injection of bacteria and reported that the same degree of response followed in typhoid immune animals when injected with *B. coli* as when typhoid bacilli were administered. In part, this is also stated to be the experience of Jobling and Peterson. The whole question is so deeply involved with important problems of therapy and was so especially important to us in connection with our own projects to re-examine the effects of leucocytic extracts (in which we wish to substitute the intravenous for the subcutaneous

method of administration) that we thought it advisable to subject the problem of leucocytosis to further inquiry.

We submit the following protocols before we attempt to analyze them or interpret their significance.

*Experiment 1*

TYPHOID IMMUNE RABBIT 1101 Nine days after the last injection		NORMAL RABBIT 1104	
Injected on November 1 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 14,000		Injected on November 1 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 12,400	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
1	2,000	1	4,400
2	900	2	2,300
4	1,600	4	5,000
6	2,800	6	6,200
8	8,600	8	18,500
10	9,200		
12	10,300	This rabbit looked very sick after injection. Respiration was quick. After pricking the ear with a needle it continued to bleed for one and one-half hours. It died eight and one-fourth hours after injection.	
14	16,600		
16	14,800		
18	11,400		
20	15,200		
22	18,700		
24	14,600		

*Experiment 2*

TYPHOID IMMUNE RABBIT 328 Nineteen days after the last injection		TYPHOID IMMUNE RABBIT 52 Nineteen days after the last injection		NORMAL RABBIT 237		NORMAL RABBIT 1101	
Injected on September 6 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 11,500		Injected on August 6 with one-tenth agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 10,400		Injected on August 6 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 11,000		Injected on August 6 with one-tenth agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 10,300	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
18	20,000	18	26,600	18	19,800	18	18,600
20	25,000	20	10,100	20	19,600	20	20,300
22	37,400	22	13,100	22	55,600	22	20,600
24	22,400	24	14,600	24	35,800	24	19,100
26	27,500	26	14,900	26	29,400	26	16,200
28	30,700	28	18,500	28	28,100	28	23,400
						29	21,300

In both of these experiments it is apparent that the typhoid immune rabbits did not react either more rapidly or more strongly than did the normal to the injection of the bacteria. Indeed in the first injection in rabbit 237, in which  $\frac{1}{2}$  an agar slant of live typhoid bacilli was given, it is interesting to note that the rabbit died in spite of the high leucocytic count, although rabbit 328, which reacted apparently less vigorously, survived. In this experiment, however, the counts were not begun until eighteen hours after the injection, a criticism we make also of most of the protocols, given by McWilliams and which we thought at first might be responsible for results different from those of Gay and Claypole. In most of the subsequent work, as will be seen by the protocols, the counts were started earlier and carried on every two hours as indicated.

The protocols which follow represent further counts made on normal and typhoid immune animals.

In the following protocols it is noticeable that, although we never obtained the very high counts reported by Gay and Claypole, there is nevertheless a distinct difference in favor of the typhoid immune rabbits. We defer the discussion of these results also until we have submitted further experiments.

*Experiment 3*

TYPHOID IMMUNE RABBIT 328 Seventeen days after the nineteenth or last injection		NORMAL RABBIT 329	
Injected on October 10 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 8,600		Injected on October 10 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 14,000	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
2	3,000	2	2,400
4	4,400	4	3,100
6	14,100	6	2,900
8	31,400	8	4,500
10	47,000	10	8,100
12	41,000	12	8,500
14	39,600	14	16,200

## Experiment 4

RABBIT 597				NORMAL RABBIT 319		TYPHOID IMMUNE RABBIT 597
Normal. (Experiment 1.) Same rabbit before immunization		Immunized against <i>B. typhosus</i> seven days after the last injection, 54 days after the last experiment		Injected on October 23 with one-tenth agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 9,200		Injections
Injected on August 30 with one-tenth agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 11,000		Injected on October 23 with one-tenth agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 12,500				
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	
7		7	6,400	7	2,000	1. August 30. 1-10 slant live <i>B. typhosus</i> intrav. injected. 2. September 28. 1-5 slant live <i>B. typhosus</i> intrav. injected. 3. October 3. 1-5 slant live <i>B. typhosus</i> intrav. injected. 4. October 23. 1-10 slant live <i>B. typhosus</i> intrav. injected.
9		9	18,000	9	4,800	
11		11	26,000	11	9,800	
13		13	20,600	13	7,600	
15		15	37,200	15	9,400	
17		17	39,600	17	17,600	
18	17,200	18		19		
19		19	18,000	20	14,800	
20	18,100	20		21		
21		21	29,200	22	13,600	
22	15,200	22		23		
23		23	24,300	24	16,200	
24	19,100	24		25		
25		25	17,000	26	17,500	
26	20,500	26		27		
27		27		28		
28	22,000	28				

## Experiment 5

TYPHOID IMMUNE RABBIT 328		NORMAL RABBIT 365	
Nine days after the last (15th) injection			
Injected on August 28 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 12,600		Injected on August 28 with half agar slant of live <i>B. typhosus</i> . Before injection the leucocytic count is 9,300	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
18	31,400	18	10,200
20	33,000	20	10,800
22	40,100	22	16,660
24	40,600	24	18,200
26	38,000	26	15,400
28	39,100	28	19,800

*Experiment 6*

TYPHOID IMMUNE RABBIT 1101 Twenty-six days after last injection		TYPHOID IMMUNE RABBIT 405 Forty-three days after last injection		NORMAL RABBIT 521	
Injected on December 5 with one-tenth slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 10,400		Injected on December 5 with one-tenth slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 5400		Injected on December 5 with one-tenth slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 8000	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
8	19,200	8	13,200	8	19,400
10	<b>45,000</b>	10	<b>60,000</b>	10	18,600
12	13,000	12	22,200	12	<b>42,000</b>
14	12,000	14	9,400	14	20,600
17	23,000	17	9,600	17	28,000
19	<b>24,000</b>	19	9,200	19	<b>47,000</b>
22	18,400	22	<b>33,600</b>	22	37,800
24	20,000	24	18,000	24	28,000
36	9,600	36	11,800	36	18,000

The protocols immediately following represent counts made on rabbits immunized with *B. prodigiosus*, *B. dysenteriae* Flexner and Strong, and *B. coli communior*, together with comparative counts made at the same time on normal controls.

*Experiment 7*

B. PRODIGIOSUS, IMMUNE RABBIT 466 Before injection 7200 injected with one-tenth live <i>B. prodigiosus</i> . Agglutination-1-4,000		NORMAL RABBIT 497 Before injection 17,000 injected with live <i>B. prodigiosus</i>	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
6 ( 6.00 a.m.)	5,200	6 ( 6.15 a.m.)	5,000
8 ( 8.00 a.m.)	9,000	8 ( 8.15 a.m.)	15,200
10 (a.m.)	18,200	10 (10.15 a.m.)	22,400
12 (12.00 a.m.)	16,400	12 (12.15 a.m.)	21,000
14 ( 2.00 p.m.)	19,200	14 ( 2.15 p.m.)	<b>36,000</b>
16 ( 4.00 p.m.)	18,000	16 ( 4.15 p.m.)	28,400
18 ( 6.00 p.m.)	16,000	18 ( 6.15 p.m.)	20,400
20 ( 8.00 p.m.)	<b>24,000</b>	20 ( 8.15 p.m.)	26,000
22 (10.00 p.m.)	17,400	22 (10.15 p.m.)	27,500
24 (12.00 p.m.)	9,800	24 (12.15 p.m.)	32,390
26 ( 2.00 a.m.)	9,500	26 ( 2.15 a.m.)	26,000

## Experiment 8.

B. PRODIGIOSUS IMMUNE RABBIT 466 Ten days after last injection		NORMAL RABBIT 497		B. PRODIGIOSUS, RABBIT 466	
Injected on November 9 with one-tenth agar slant of live <i>B. prodigiosus</i> . Before injection the leucocyte count is 7200		Injected on November 9 with one-tenth agar slant of live <i>B. prodigiosus</i> . Before injection the leucocyte count is 17,000		Weights	Injections
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes		
6	5,200	6	5,000	Aug. 2. 2420	1. Aug. 2. 1-50 slant dead <i>B. prodigiosus</i> intravenous injection.
8	9,000	8	15,200	Aug. 4. 2230	
10	18,200	10	22,400	Aug. 5. 2200	
12	16,400	12	21,000	Aug. 7. 1980	
14	19,200	14	36,000	Aug. 9. 1920	2. Aug. 17. 1-50 slant dead <i>B. prodigiosus</i> intravenous injection.
16	18,600	16	28,400	Aug. 10. 1915	
18	16,000	18	20,400	Aug. 14. 1730	
20	24,000	20	26,000	Aug. 16. 1810	
22	17,400	22	27,500	Aug. 17. 1810	3. Aug. 29. 1-20 slant dead <i>B. prodigiosus</i> , intravenous injection.
24	9,800	24	32,390	Aug. 24. 1630	
26	9,500	26	26,000		
				Sept. 5. 1545	4. Sept. 28. 1-20 slant dead <i>B. prodigiosus</i> intravenous injection.
				Sept. 21. 1825	
				Sept. 28. 1970	
				Oct. 3. 1990	5. Oct. 3. 1-10 slant dead. <i>B. prodigiosus</i> intravenous injection
				Oct. 11. 1870	
				Oct. 16. 1820	
					6. Oct. 16. 1-5 slant dead <i>B. prodigiosus</i> intravenous injection.
				Oct. 30. 1720	
				Nov. 9. 1790	7. Oct. 30. 1-10 slant live <i>B. prodigiosus</i> intravenous injection.
					Nov. 9. 1-10 slant live <i>B. prodigiosus</i> intravenous injection.

Titre = 1 = 4, 000.

*Experiment 9*

DYSENTERY (FLEXNER), IMMUNE RABBIT 157		NORMAL RABBIT 158	
Forty-six days after the 15th of last injection, on August 31. Injected with leucocytes on August 22			
Injected on October 16 with one-half agar slant of live <i>B. dysenteriae</i> (Flexner). Before injection the leucocyte count is 10,400		Injected on October 16 with one-half agar slant of live <i>B. dysenteriae</i> (Flexner). Before injection the leucocyte count is 9600	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
2	3,600	2	2,800
4	6,200	4	2,500
6	13,800	6	3,100
8	17,200	8	5,200
10	16,700	10	5,800
12	23,000	12	<b>12,800</b>
14	<b>24,000</b>	14	10,600
16	18,200	16	15,600
18	29,600	18	16,000
20	35,100	20	18,400
22	<b>42,200</b>	22	17,200
24	28,200	24	16,100
26	31,000	26	<b>24,700</b>
28	15,700	28	16,400
30		30	

*Experiment 10*

DYSENTERY (STRONG), IMMUNE RABBIT 168		NORMAL RABBIT 335		NORMAL RABBIT 502		DYSENTERY (FLEXNER) IMMUNE RABBIT 157	
Sixteen days after last (seventeenth) injection						Six days after last (seventeenth) injection	
Injected on August 22 with one-twentieth slant agar of dead <i>B. dysenteriae</i> . Before injecting the leucocyte count is 4000		Injected on August 22 with one-twentieth agar slant of dead <i>B. dysenteriae</i> (Strong). Before injecting the leucocyte count is 9000		Injected on August 22 with 10 cc. leucocytic extract. Before injecting the leucocyte count is 11,600		Injected on August 22 with 10 cc. leucocyte extract. Before injection count is 11,000	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
18	8,000	18	25,600	18	<b>37,800</b>	18	27,600
20	14,200	20	26,200	20	37,000	20	22,800
22	13,600	22	<b>30,600</b>	22	29,200	22	<b>31,000</b>
24	16,500	24	23,200	24	31,600	24	26,500
26	19,400	26	26,800	26	25,900	26	18,500
28	<b>23,200</b>	28	18,400	28	29,000	28	18,800

*Experiment 11*

COLON (IOR) IMMUNE RABBIT 169 5 days after last (twelfth) injection		NORMAL RABBIT 339		NORMAL RABBIT 999		COLON (IOR) IMMUNE RABBIT 170 Five days after last (twelfth) injection	
Injected on August 17 with one-twentieth agar slant of dead <i>B. coli</i> (ior). Before in- jection the leucocyte count is 8200		Injected on August 17 with one-twentieth agar slant dead <i>B. coli</i> (ior). Before injection the leucocyte count is 10,000		Injected on August 17 with 10 cc. leucocyte extract. Before in- jection the leucocyte count is 13,200		Injected on August 22 with 10 cc. leucocyte extract. Before in- jection the leucocyte count is 8600	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
18	15,000	18	17,400	18	21,200	18	30,000
20	15,400	20	14,800	20	16,400	20	16,400
22	13,600	22	21,400	22	18,800	22	22,000
24	14,800	24	18,200	24	18,600	24	17,000
26	11,400	26	21,400	26	16,600	26	16,800
28	18,200	28	22,800	28	14,600	28	12,600

*Prodigiosus* not only does not show values which favor the immune animals, but rather the reverse, in that more energetic and more rapid leucocytosis occurs in the normal control. The two dysenteriae experiments 9-10, are similar, and there is only one protocol in this series in which the counts were in favor of the immune animals, and that is in the case of the last animal, rabbit 170 of the *Colon* series. Moreover it should be noted that the difference both in time and maximum number throughout the series are relatively slight and hardly greater than, in our experience, might be expected as likely to occur between individual animals, normal or immune.

The foregoing experiments were all done with Gram-negative bacilli. In view of the fact that in these organisms immunological conditions, especially as regards the circulating leucocytes, are quite different from those encountered in the cases of infection with the Gram-positive cocci, it seemed desirable to carry out a few experiments with streptococci and staphylococci. These follow.



### Experiment 12

STREPTOCOCCUS IMMUNE RABBIT 296		NORMAL RABBIT		STREPTOCOCCUS IMMUNE RABBIT 296
Injected on October 19 with 1 K of streptococcus. Before injection the leucocyte count is 8400		Injected on October 19 with 1 K of streptococcus. Before injection the leucocyte count is 13,200		Injections
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	
7	22,000	7	13,200	1. Aug. 22. 1 K intravenous injection
8	21,800	8	10,100	2. Aug. 29. 2 K intravenous injection
10	20,900	10	11,800	3. Sept. 5. 2 K intravenous injection
12	16,200	12	14,700	4. Sept. 12. 2 K intravenous injection
14	10,200	14	15,000	5. Sept. 16. 1 K intravenous injection
16	10,400	16	8,300	6. Sept. 20. 2 K intravenous injection
18	13,400	18	11,000	7. Oct. 4. 2 K intravenous injection
20	8,600	20	10,800	8. Oct. 12. 1 K intravenous injection
		22		Oct. 19. 1 K intravenous injection
		24		
		26		
		28		

### Experiment 13

STREPTOCOCCUS IMMUNE RABBIT 436*		NORMAL RABBIT 437		STREPTOCOCCUS IMMUNE RABBIT 436
Seven days after last injection. The rabbit was thin and sick before injection				
Injected on October 19 with $\frac{1}{4}$ F.K. of streptococcus. Before injection the leucocyte count is 8200		Injected on October 19 with $\frac{1}{4}$ F.K. of streptococcus. Before injection the leucocyte count is 9400		
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	
7	4,500	7	3,200	1. Aug. 22. 1 K intravenous injection
8	4,800	8	5,400	2. Aug. 29. 2 K intravenous injection
10	4,400	10	8,000	3. Sept. 5. 2 K intravenous injection
12	6,200	12	10,600	4. Sept. 12. 2 K intravenous injection
14	4,600	14	9,500	5. Sept. 16. 1 K intravenous injection
16	4,500	16	11,400	6. Sept. 20. 2 K intravenous injection
18	4,200	18	13,200	7. Oct. 4. 2 K intravenous injection
20	4,600	20	12,000	8. Oct. 12. 1 K intravenous injection
22		22		Oct. 19. $\frac{1}{4}$ FK intravenous injection
24		24		
26		26		
28		28		

\* This rabbit sick. Has been losing weight.

*Experiment 14*

STAPHYLOCOCCUS PYOGENES AUREUS IMMUNE RABBIT 953		NORMAL RABBIT 127	
Before injection 7000.	To live Staphylococcus pyogenes aureus. Agglutination-1-200	Before injection 18,000.	To live Staphylococcus pyogenes aureus
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
6 ( 6.30)	8,600	6 ( 6.45)	9,400
8 ( 8.30)	20,400	8 ( 8.45)	33,200
10 (10.30)	53,200	10 (10.45)	39,000
12 (12.30)	22,100	12 (12.45)	28,800
14 ( 2.30)	14,000	14 ( 2.45)	21,600
16 ( 4.30)	16,100	16 ( 4.45)	16,800
18 ( 6.30)	16,200	18 ( 6.45)	29,400
20 ( 8.30)	18,400	20 ( 8.45)	35,500
22 (10.30)	28,000	22 (10.45)	24,800
24 (12.30)	24,900	24 (12.45)	28,900
26 ( 2.30)	19,800	26 ( 2.45)	25,100

*Experiment 15*

STAPHYLOCOCCUS IMMUNE RABBIT 946 Seven days after last injection		NORMAL RABBIT 883		STAPHYLOCOCCUS IMMUNE RABBIT 946	
Injected on October 23 with one-tenth agar slant of live <i>Staphylococcus pyogenes aureus</i> . Before injection the leucocyte count is 11,600		Injected on October 23 with one-tenth agar slant live <i>Staphylococcus pyogenes aureus</i> . Before injection the leucocyte count is 9000		Injections	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes		
7	85,000	7	18,600	1. August 11. 1-100 agar slant dead Staphylococcus pyogenes aureus intravenous injection	
9	37,600	9	26,900	2. August 29. 1-50 agar slant dead Staphylococcus pyogenes aureus intravenous injection	
11	43,200	11	28,700	3. September 28. 1-25 agar slant dead Staphylococcus pyogenes aureus intravenous injection	
13	33,400	13	32,000	4. October 3. 1-10 agar slant dead Staphylococcus pyogenes aureus intravenous injection	
15	3,600	15	27,600	5. October 16. 1-5 agar slant dead Staphylococcus pyogenes aureus intravenous injection	
17	19,000	17	16,100	October 23. 1-10 agar slant live Staphylococcus pyogenes aureus intravenous injection	
19	18,400	19	25,400		
21	15,600	21	32,800		
23	17,800	23	28,600		
5	15,300	25	26,000		
27		27			

## Experiment 16

STAPHYLOCOCCUS, IMMUNE RABBIT 953		NORMAL RABBIT 127		STAPHYLOCOCCUS IMMUNE RABBIT 953
Injected on November 9 with one-tenth agar slant of live <i>Staphylococcus pyogenes aureus</i> . Before injection the leucocyte count is 7,600		Injected on November 9 with one-tenth agar slant live <i>Staphylococcus pyogenes aureus</i> . Before injection the leucocyte count is 18,000		Injections
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	
6	8,600	6	9,400	1. August 11. 1-100 agar slant dead <i>Staphylococcus pyogenes aureus</i> intravenous injection
8	20,400	8	33,200	2. August 29. 1-50 agar slant dead <i>Staphylococcus pyogenes aureus</i> intravenous injection
10	53,200	10	39,000	3. September 28. 1-25 agar slant dead <i>Staphylococcus pyogenes aureus</i> intravenous injection
12	22,100	12	28,800	4. October 3. 1-10 agar slant dead <i>Staphylococcus pyogenes aureus</i> intravenous injection
14	14,000	14	21,000	5. October 16. 1-5 agar slant dead <i>Staphylococcus pyogenes aureus</i> intravenous injection
16	16,100	16	16,800	6. October 23. $\frac{1}{2}$ agar slant dead <i>Staphylococcus pyogenes aureus</i> intravenous injection
18	16,200	18	29,400	7. October 30. 1-10 agar slant live <i>Staphylococcus pyogenes aureus</i> intravenous injection
20	18,400	20	35,500	November 9. 1-10 agar slant live <i>Staphylococcus pyogenes aureus</i> intravenous injection
22	28,000	22	24,800	
24	24,960	24	28,900	
26	19,800	26	25,100	

Titre = 1:200.

Experiments 12 to 16 inclusive show in general that animals immunized with Gram-positive cocci respond to reinjection with the homologous bacteria by a leucocytosis which is numerically higher and in general more rapid in development. In experiment 13 it is quite noticeable how important is the con-

dition of the rabbit apart from the degree of immunity. Rabbit 436 in this experiment had received eight preliminary intravenous injections of streptococci but was losing weight, and was generally in poor condition. In consequence the injection of another dose of streptococci led to a leucopenia, in spite of which the rabbit did not die. This is a point which we will take up in the discussion below.

In all the reported experiments, the injection of homologous bacteria into immune rabbits was controlled only by the injection of the same bacteria into normal animals. In view of the work of McWilliams it seemed desirable to carry out a few experiments on the injection of bacteria other than those used for immunization, into immunized animals. In the following experiment (no. 17) bacillus prodigiosus was injected into a prodigiosus immune animal, bacillus typhosus into a similar animal and bacillus prodigiosus into a normal animal.

*Experiment 17*

B. PRODIGIOSUS, IMMUNE RABBIT 466		B. PRODIGIOSUS, IMMUNE RABBIT 237		NORMAL RABBIT 111	
Twenty-six days after the last injection has had nine injections of <i>B. prodigiosus</i>		Twenty-six days after the last injection has had five injections of <i>B. prodigiosus</i>			
Injected on December 5 with one-tenth slant of <i>B. prodigiosus</i> . Before injection the leucocyte count is 8000		Injected on December 5 with one-tenth slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 9500		Injected on December 5 with one-tenth slant of live <i>B. prodigiosus</i> . Before injection the leucocyte count is 7800	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
8	8,800	8	7,400	8	15,200
10	10,600	10	10,000	10	<b>67,600</b>
12	<b>12,800</b>	12	10,400	12	28,000
14	8,400	14	7,800	14	32,000
17	8,000	17	<b>18,200</b>	17	25,000
19	8,600	19	11,400	19	35,400
22	<b>13,800</b>	22	<b>14,800</b>	22	27,600
24	11,000	24	10,200	24	38,000
36	8,000	36	7,000	36	28,000

In experiment 18 typhoid bacilli and streptococcus were injected into typhoid immune and streptococcus immune animals, and into normal control in the same way.

*Experiment 18*

TYPHOID, IMMUNE RABBIT 7		TYPHOID IM- MUNE RABBIT 328		NORMAL CON- TROL RABBIT 1056		STREPTO- COCCI, IM- MUNE RABBIT 34		STREPTOCOCCUS, IMMUNE RABBIT 491		NORMAL CON- TROL RABBIT 1044	
Injected with one-tenth slant of live <i>B. typho-</i> <i>sus</i> . Weight decreased (1565). Count = 4000		Injected with 2 cc. twenty- four hour broth cul- ture of live <i>streptococci</i> . Count = 7400		Injected with one-tenth slant live <i>B.</i> <i>typhosus</i> . Weight = 1150. Count = 5000		Injected with 2 cc. twenty- four hour broth cul- ture of live <i>strepto-</i> <i>cocci</i> . Weight = 1815. Count = 10800		Injected with one-tenth slant of live <i>B. typhosus</i> . Weight = 1930. Count = 7000		Injected with 2 cc. twenty- four hour broth cul- ture live <i>streptococci</i> . Weight = 1300. Count = 7000	
8— (6gr.)	6,800	8—	7,000	8—	6,200	8—	7,400	8—	6,800	8—	3,200
10—	22,800	10—	6,000	10—	10,000	10—	14,000	10—	10,800	10—	6,400
12—	7,200	12—	8,600	12—	18,000	12—	9,200	12—	20,400	12—	5,000
14—	8,400	14—	8,200	14—	15,800	14—	9,000	14—	20,000	14—	11,200
17—	12,000	17—	8,000	17—	17,000	17—	20,200	17—	34,400	17—	11,000
19—	13,400	19—	13,200	19—	45,000	19—	12,400	19—	27,600	19—	6,000
22—	14,400	22—	10,000	22—	26,000	22—	8,200	22—	23,200	22—	5,400
24—	17,600	24—	11,000	24—	27,200	24—	11,200	24—		24—	3,800
25—	8,800	25—									

In the second experiment there is a slight indication of greater leucocytosis when the homologous bacteria are injected into the immune animal. In the first the normal animal responded most actively.

Throughout our work it has seemed to us that an important factor in determining the response of animals to reinjection was to be found in the interval between injections, that is, too rapid injection might add further injury before the animal had fully recovered from the preceding dose. This is very naturally a complement of the factor found in the condition of the animal. Indeed, throughout such work with animals there has been too little attention paid to the individual variation in the condition of the subject of experimentation, a matter which, were we applying specific therapy to human beings, would of course be regarded as paramount.

The following three experiments were planned to take the reinjection intervals into particular consideration.

### Experiment 19

TYPHOID IMMUNE RABBIT 328 Eighteen days after last injection (sixteenth)		NORMAL RABBIT 938 (Weight 1555)		TYPHOID IMMUNE RABBIT 450 Two days after last injection		TYPHOID IMMUNE RABBIT 52 Five days after last injection	
Injected on July 5 with one-half agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 12,000 (two hours before injection)		Injected on July 20 with 5 cc. of leucocyte extract. Before injection the leucocyte count is 11,800 (one hour before injection)		Injected on July 21 with one-half agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 14,800		Injected on July 22 with agar slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 13,500	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
$\frac{1}{2}$	3,100	$\frac{1}{2}$	13,500	$\frac{1}{2}$	2,800		
		1	18,000	1	4,100		
		$1\frac{1}{2}$	14,570				
		2	12,800				
		$2\frac{1}{2}$	15,250				
		3	19,000				
		4	22,400				
5	5,000	5	16,400				
		16	15,400	16	20,740		
		17	11,000	17	13,800	17	33,540
		18	11,600	18	14,000	18	45,800
		19	17,800	19	18,400	19	40,000
		20	14,100	20	19,000	20	40,500
		21	15,740	21	11,000	21	39,800
22	35,000	23	18,800	22	11,400	22	39,800
		26	20,000	23	15,000	23	33,000
				24	17,800	24	24,400
				25	19,300	25	28,000
				26	15,500	26	27,500
				27	15,000	27	37,300
				28	14,000	28	34,800

### Experiment 20

B. PRODIGIOSUS IMMUNE RABBIT 466 Six days after last injection		B. PRODIGIOSUS IMMUNE RABBIT 273 Forty-one days after last injection	
Injected on December 11 with one-tenth slant of live <i>B. prodigiosus</i> . The leucocyte count before injection is 10,000		Injected on December 11 with one-tenth slant of live <i>B. typhosus</i> . The leucocyte count before injection is 7200	
Hours after injection	Number of leucocytes	Hours after injection	Number of leucocytes
8	16,200	8	7,000
10	22,400	10	13,800
12	18,600	12	20,000
14	32,000	14	20,600
17	19,800	17	20,800
19	17,800	19	19,000
22	16,000	22	22,400
24	10,400	24	20,600

*Experiment 21*

TYPHOID IMMUNE RABBIT 1101 Six days after last injection		TYPHOID IMMUNE RABBIT Four days after last injection		TYPHOID IMMUNE RABBIT 597 Thirty-two days after last injection	
<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>
8	10,400	8	5,000	8	6,800
10	12,000	10	<b>16,000</b>	10	<b>24,000</b>
12	<b>53,200</b>	12	7,400	12	16,000
14	34,000	14	11,200	14	15,600
17	36,000	17	12,400	17	20,000
19	28,800	19	<b>17,000</b>	19	18,000
22	<b>43,400</b>	22	16,000	22	<b>28,600</b>
24	29,000	24	15,000	24	22,200

We have done several experiments on the influence of the dose injected but do not insert these as they merely show what one would naturally expect; that an optimum dose must be found which must sufficiently stimulate the reaction but which will not intoxicate the animals to such an extent that the response is completely inhibited.

*Experiment 22*

TYPHOID IMMUNE RABBIT 597 Seven days after last injection. Twenty-four days after last experiment		NORMAL RABBIT 1104		TYPHOID IMMUNE RABBIT 328 Thirty-seven days after last injection		NORMAL RABBIT 440	
Injected on November 16 with 8 cc. of leucocytic extract. Before injection the leucocyte count is 12,600		Injected on November 16 with 8 cc. of leucocyte extract. Before injection the leucocyte count is 10,800		Injected on November 16 with one-half slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 6800		Injected on November 16 with one-half slant of live <i>B. typhosus</i> . Before injection the leucocyte count is 11,700	
<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>
2	10,000	2	16,800	2	2,400	2	1,000
4	10,600	4	17,000	4	2,000	4	1,200
6	16,400	6	16,000	6	6,000	6	1,700
8	<b>18,200</b>	8	14,800	8	12,300	8	3,000
10	15,800	10	<b>18,600</b>	10	29,000	10	Died
12	13,200	12	16,400	12	29,200	12	
14	9,400	14	16,200	14	35,800	14	
16	15,600	16	9,600	16	24,200	16	
18	14,000	18	12,400	18	20,200	18	
20	13,800	20	10,200	20	<b>37,000</b>	20	

*Experiment 23*

TYPHOID IMMUNE RABBIT 597		TYPHOID IMMUNE RABBIT 328		NORMAL RABBIT 440		NORMAL RABBIT 1104	
8 cc. leucocyte extract (thick). 12,600		One-half slant <i>B. typho-</i> <i>sus.</i> Seven days after last injection. 10,800		One-half slant <i>B. typho-</i> <i>sus.</i> 6800		8 cc. leucocyte extract (thick). 11,700	
<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>	<i>Hours after injection</i>	<i>Number of leucocytes</i>
2 ( 1.45)	10,000	2	2,400	2	1,000	2	16,800
4 ( 3.45)	10,600	4	2,000	4	1,200	4	17,000
6 ( 5.45)	16,400	6	6,000	6	1,700	6	10,000
8 ( 7.45)	18,200	8	12,300	8	3,000	8	14,800
10 ( 9.45)	15,800	10	29,000	10	Died	10	18,000
12 (11.45)	13,200	12	29,200	12		12	16,400
14 ( 1.45)	9,400	14	35,800	14		14	16,200
16 ( 3.45)	15,000	16	24,200	16		16	9,000
18 ( 5.45)	14,000	18	20,200	18		18	12,400
20 ( 7.45)	13,800	20	37,000	20		20	10,200
22 ( 9.45)		22		22		22	
24 (11.45)		24		24		24	
26 ( 1.45)		26		26		26	
28 ( 3.45)		28		28		28	
30 ( 5.45)		30		30		30	

Since our work was begun largely with the purpose of determining whether or not specific vaccine therapy was more promising as a therapeutic measure than the injection of non-specific leucocytosis stimulating substances with special consideration of leucocyte extract, we also carried out counts on normal and immunized animals, injected with leucocytic extracts.

Such experiments, though not representing all of those done, are illustrated by experiments 22-23.

## DISCUSSION

A critical scrutiny of the experiments just reported reveals the following facts.

When homologous Gram-negative bacilli are injected into immunized animals there seems to be a definitely higher leucocytosis in the immunized animals than in normal controls similarly treated. The contrasts in our experiments however, were nothing like as striking as those reported by Gay and Clay-



pole. Indeed the contrast in general is so slight and so irregular that in the case of the Gram-negative bacilli we were at first inclined to agree with McWilliams. There was, however, a sufficiently definite difference in an average of many counts to convince us that this was more than coincidence.

In the case of the Gram-positive cocci there was a more marked difference, in that, the immunized animals reacted more promptly and very much more energetically than did the normal animals.

It seems reasonably clear, then, that an animal reacts more energetically as far as its mobilization of leucocytes is concerned when reinjected than does a normal animal treated with the same variety and quantity of bacteria.

The reaction is dependent upon a number of factors, chief among which are: (1) the condition of the animal (loss of weight, etc.); (2) the amount of bacteria injected, and (3) the interval between injections. These factors all very naturally signify the necessity of avoiding too profound an intoxication of the animal. This is very well illustrated in some of the experiments with typhoid bacilli and especially in one of the experiments with streptococci, in which there was no leucocytic response in a highly immunized streptococcus animal which was in poor general condition, whereas, the normal control animal reacted vigorously to the same dose.

When immune animals are treated with heterologous bacteria; that is, when prodigious bacilli or colon bacilli are injected into typhoid immune animals and vice versa, there seems to be no specific difference in response. That is, the injection of colon bacilli into typhoid animals has shown no marked difference in leucocytic response from that observed when typhoid bacilli were injected into a typhoid animal. In this our figures correspond with those of McWilliams. They are also in keeping with the clinical experience of Kraus, Ichikawa, and others which have been mentioned above.

The injection of leucocytic extract does not arouse as vigorous a leucocytic response as does the injection of bacillary protein. However, the preliminary leucopenia is either very slight or entirely absent when this substance is injected, and the leucocytes

are usually about doubled when ordinary doses of the extract are given intravenously.

In reading these facts superficially they at first seem to be contradictory in significance, in as much as specificity seems to exist in the fact that typhoid immune rabbits or streptococcus immune rabbits respond somewhat more vigorously than do normal controls injected with the same substance. Nevertheless we believe that certain observations made several years ago by one of the writers with Dr. Dwyer serve to cast some light upon these relations. We were dealing at the time with the poisons which were formed when typhoid bacilli were allowed to react with active normal guinea-pig serum. These poisons have been spoken of variously as, "anaphylatoxins," "proteotoxins," or "serotoxins," and the mechanism of their production is still to be finally settled, although we are inclined to take the view of Jobling and Peterson. However, the mechanism has no special bearing on the problem before us at present. That they can be formed in the animal body, that is intraperitoneally, has been shown by Friedberger and we believe that there is much reason for regarding them as identical with the so-called aggressins of Bail. It is at least very likely that these poisons are of the same nature as those formed when bacteria are injected into the animal body and play a part in the rapid intoxication which follows the injection of such organisms as typhoid and colon bacilli into rabbits. It has been shown that such substances injected into an animal induce leucopenia.

It was shown by Dwyer and writer that the proteotoxin or serotoxin when injected into the animal body together with sublethal doses of various bacteria, would lead to the death of the animal; i.e. by making it possible for the original sublethal dose of the microörganism to kill. It was found that this action was non-specific in that the aggressin-like, or virulence-enhancing properties were exerted by poisons made with typhoid bacilli indiscriminately upon typhoid bacilli and staphylococci and we expressed the belief that the mechanism of such action was dependent upon a general poisoning of the animal in the course of which the mobilization of leucocytes, among other

functions, was depressed. We were further able to show, as did also Jobling and Peterson and others, that although animals do not attain immunity in the ordinary sense to these poisons, nevertheless, they may attain a relative tolerance which appears after the sixth day, lasts until the fiftieth or sixtieth, and enables the animal to withstand one and one-half to two lethal doses.

On the basis of this we think we may coördinate the observations recorded above. When an animal is injected with Gram-negative bacilli, in the presence of which this poison is formed with relative ease, the animal is intoxicated, which accounts for the preliminary leucopenia, and then reacts vigorously if the dose has been moderate and the toxemia easily overcome. If the dose is too large and the toxemia too profound, or the injection cumulative in so far as it has followed too soon upon another injection, the reaction is less vigorous.

Since a relative tolerance to these poisons is acquired and since this tolerance is non-specific, one would expect on the one hand that immunized animals would be less easily intoxicated than normal animals, and that furthermore animals treated with bacteria beforehand would react with less injury and more vigorous leucocytic mobilization both to homologous and heterologous bacteria, than would normal controls to the same dose.

This reasoning would go far to explain why leucopenia accompanying the profound intoxication of an animal or a human being with such organisms as the typhoid bacillus, and also give a clue to the reason for the habitual leucocytosis accompanying Gram-positive coccus infection. It would also explain why diseases like typhoid fever have been non-specifically influenced by the injection of paratyphoid bacilli, (Ichikawa) colon bacilli, (Kraus) and such substances as the albumoses, etc., recommended by Jobling and Peterson.

The only weak link in this chain of reasoning is the uncertainty to which we must confess concerning the identity of the poisons made in vitro and those appearing in animal bodies when bacteria are injected. This question is one which we are actively investigating but it will seem at least likely, from the work of

Friedberger, Jobling and his collaborators, and others, that these poisons have much in common. Whether they are formed in the circulation or on the cells, whether they are derivatives of the bacterial antigen or result from auto-digestion of the plasma, these are points of great importance but not directly pertinent to this particular discussion.

Furthermore we wish to emphasize the fact that we do not regard these leucocytic reactions as necessarily the sole factors in the resistance of animals. It may well be that mobilization of ferments and the more slowly acquired functional changes in fixed tissue cells are more important. But in this work we are using them as easily measured indicators of resistance that signify the readiness with which the animal can command its emergency apparatus for overcoming the insult.

As far as the work has direct bearing on the leucocytic extract of Hiss it is apparent that although these substances do not produce the extreme reaction which follows the injection of bacterial proteins, they do not on the other hand induce so severe a leucopenia. With this in mind, and remembering the unquestionably favorable results obtained by Hiss in animals and the occasional favorable results obtained by Hiss and one of us in human beings, we are inclined to think that it would be worth while again to test out these substances as therapeutic agents by making them isotonic and using them intravenously instead of subcutaneously as heretofore.

# THE INTRACUTANEOUS ABSORPTION OF ANTIGEN

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That the body is capable of reacting to parenterally introduced antigenic substances has been amply proven, but as yet there has been no satisfactory explanation of the mechanism of the interaction, that is, it has not been conclusively determined whether antigen is eliminated as antigen or whether it enters into the metabolic processes and becomes utilized or denatured. It can not be assumed that identical processes follow the introduction of different antigens or even of the same antigen when injected by different routes. In fact, opinion differs very widely as to what happens when a given antigen is introduced by a single route. Further complications arise through the fact that animals receiving antigen may, as a result of inherent peculiarities or experimental procedure, be endowed with a variety of metabolic forces, which in individual cases may so combine as to give a variety of results. A study of these problems is of considerable importance since they are directly concerned with the subjects of anaphylaxis and immunity.

The ordinary methods of introducing antigen have led to different views as to its disposition, but these procedures can not be sufficiently controlled to afford very definite proof. In the intracutaneous reaction, however, we have a method which is particularly well adapted for determining the processes involved in the local response of the body to foreign protein. While the cutaneous reaction has been extensively used for clinical purposes, little emphasis has been placed upon the mechanism through which the reactions are obtained.

In the literature we find but few references to this particular point. Fukuhara<sup>1</sup> working with normal pigs and pigs sensitized to sera from several sources showed that a specific reaction in the case of the sensitized pigs could be obtained with the homologous antigen, although the reaction was slight in degree. The symptoms produced are ascribed by him to the interaction of antigen and cellular antibody. The work of other authors has been directed toward drawing a parallelism between intracutaneous reactions and absorption phenomena. They have not approached the problem by determining the presence of the specific antigens in the tissue. It has been our purpose to demonstrate the persistence of native unaltered antigen in the tissues under a variety of experimental conditions. And, while the work here reported falls far short of presenting an explanation of the mechanism of cutaneous reactions, a few of the facts disclosed in the experimentation possess a certain interest.

The preliminary work was concerned with demonstrating the presence of antigen in the skin of normal and sensitized guinea-pigs after intracutaneous injections. As a means of detecting the antigen (normal horse serum) the anaphylactic test was employed. The procedure consisted in injecting serum into the cutaneous tissue and, after certain intervals, in removing the portions of skin into which the injections were made, grinding in a mortar with sand and a constant volume of physiological salt solution. A portion of this material was then injected intravenously into normal pigs and pigs previously sensitized to horse serum. If the sensitized pigs responded with anaphylactic shock it was considered that antigen was present in the tissue, while absence of symptoms in the normal pigs excluded the possibility of tissue toxicity.

The following protocol serves to illustrate the procedure followed and gives the necessary controls.

*Experiment I.* Pig 457 (sensitized) was injected intracutaneously with 0.5 cc. of normal horse serum. The portion of tissue was removed fifteen minutes after the injection and was ground in 2 cc. of physio-

<sup>1</sup> Ztschr. f. Immunitätsf., 1911, xi, 640.

logical salt solution, 0.5 cc. of the emulsion was injected. A 2 cc. quantity of saline was used throughout the work in preparing the tissue emulsions, and 0.5 cc. of the emulsion was always used for the intravenous injection. Thus, the amount of antigen contained in the 0.5 cc. injected intravenously was one-fourth of the total quantity present in the tissue emulsion. The time elapsing between the preparation of the emulsion and the intravenous injection never exceeded fifteen minutes. Death occurring with typical anaphylactic symptoms within fifteen minutes after the injection was construed as acute shock.

PIG	SENSITIZED WITH HORSE SERUM	INTRACUTANEOUS INJECTION	INTRAVENOUS INJECTION	RESULT
457	34 days previously 1/700 cc. subcutaneously	0.5 cc. horse serum into left side		
438	27 days 1/300 cc. subcutaneously		0.5 cc. emulsion 457—left side	Death in 7 minutes
439	27 days 1/300 cc. subcutaneously		0.5 cc. emulsion 457—right side (not injected)	No symptoms
459	34 days 1/2000 cc. subcutaneously		0.1 cc. horse serum	Death in 4 minutes
437	27 days 1/100 cc. subcutaneously		0.01 cc. horse serum	Death in 14 minutes
522	(Normal)		0.5 cc. emulsion 457—left side	No symptoms

Brief experience with the intracutaneous method of injection made it evident that it is extremely difficult to insure that all of the antigen remains in the cutaneous tissue. This is especially true when working with larger amounts (0.5 cc.) as the pressure produced has a tendency to force some of the serum out through the needle puncture. For this reason it is not to be expected that absolutely uniform results are to be obtained.

Before any quantitative relationships could be determined in regard to the persistence of antigen in the tissues it was necessary to know exactly how many minimal anaphylactic doses (M. A. D.) were contained in a definite amount of the serum injected. As we wished to approximate the borderline between

the amount of serum that would just cause anaphylaxis and the amount that was just insufficient to produce symptoms, we made careful determinations of the minimal anaphylactic dose and then used definite multiples of this quantity for intracutaneous injection. The smallest number of minimal anaphylactic doses used was 5, the largest 20. Five was chosen as the minimum, since even with no decrease in antigen content occurring in the tissues this was the smallest quantity that could be detected with certainty according to our procedure.

The question of time was also a factor in determining the quantitative relationships. Therefore tissues were removed, from pigs injected with a known number of minimal anaphylactic doses at definite intervals varying from twelve minutes to forty-eight hours. The results obtained from a series of animals are given in summarized form on pages 273 and 274.

From this experiment we can only conclude that if antigen is injected into the tissues of sensitized and normal pigs in quantities sufficient to obviate the technical errors, any differences which may exist in the persistence of antigen become apparent only after the lapse of a considerable period, and even then the differences are but slight. The errors which enter in are of course most pronounced in the case of the pigs receiving the smaller amounts of antigen since the margin of safety allowed amounts to only  $\frac{1}{4}$  minimal anaphylactic dose. Therefore any small loss assumes a disproportionately large value and any marked differences in the susceptibility of the pigs would lead to discordant results.

The similarity in reaction shown by the normal and sensitized pigs was quite contrary to our expectations. With a view to determining whether this factor of antigenic elimination would be influenced in any way whatsoever by the treatment of the animal, it was considered desirable to repeat this work introducing in addition a series of animals to which an immunizing course had been given. Accordingly, a series including immunized, sensitized, and normal animals was tested.



*Absorption test with 5 minimal anaphylactic doses of horse serum*

INTRACUTANEOUS INJECTION INTO PIG	SENSITIZED WITH HORSE SERUM	TISSUE REMOVED AFTER INTRA- CUTANEOUS IN- JECTION	PIG RECEIVING EMULSION	SENSITIZED WITH HORSE SERUM	INTRAVENOUS IN- JECTION OF 0.5 CC. TISSUE EMUL- SION FROM PIG	RESULT
		<i>hours</i>				
573	21 days 1/100 cc. subcu- taneously	2	601	21 days 1/100 cc. subcu- taneously	563	Anaphylactic death in 3 minutes
636	Normal	2	600	21 days 1/100 cc. subcu- taneously	636	Anaphylactic: lived
707	21 days 1/150 cc. subcu- taneously	3	677	28 days 1/100 cc. subcu- taneously	707	Anaphylactic: lived
749	Normal	3	727	21 days 1/1500 cc. subcu- taneously	749	Anaphylactic: death in 3 minutes
565	21 days 1/100 cc. subcu- taneously	4	616	21 days 1/100 cc. subcu- taneously	565	Anaphylactic: Reinjected in- travenously 4 minimal ana- phylactic doses after 2 days. No symptoms
641	Normal	4	615	21 days 1/100 cc. subcu- taneously	641	No symptoms. Reinjected in- travenously 4 minimal ana- phylactic doses after 2 days. No symptoms
738	21 days 1/100 cc. subcu- taneously	6	758	13 days 1/100 cc. subcu- taneously	738	Anaphylactic: death in 4 min- utes
752	Normal	6	761	13 days 1/100 cc. subcu- taneously	752	Anaphylactic: death in 4 min- utes
570	21 days 1/100 cc. subcu- taneously	12	610	21 days 1/100 cc. subcu- taneously	570	No symptoms
645	Normal	12	609	21 days 1/100 cc. subcu- taneously	645	No symptoms

*Absorption test with 10 minimal anaphylactic doses of horse serum*

INTRACUTANEOUS INJECTION INTO PIG	SENSITIZED WITH HORSE SERUM	TISSUE REMOVED AFTER INTRA- CUTANEOUS IN- JECTION	PIG RECEIVING EMULSION	SENSITIZED WITH HORSE SERUM	INTRAVENOUS IN- JECTION OF 0.5 CC. TISSUE EMUL- SION FROM PIG	RESULT
		<i>hours</i>				
574	21 days 1/100 cc. subcu- taneously	2	599	21 days 1/100 cc. subcu- taneously	574	Anaphylactic: death in 8 minutes
637	Normal	2	597	21 days 1/100 cc. subcu- taneously	637	Anaphylactic: death in 6 minutes
701	16 days 1/1000 cc. subcu- taneously	3	714	14 days 1/100 cc. subcu- taneously	701	Anaphylactic: death in 5 minutes
695	Normal	3	717	14 days 1/100 cc. subcu- taneously	695	Anaphylactic: lived
566	21 days 1/100 cc. subcu- taneously	4	614	21 days 1/100 cc. subcu- taneously	566	Anaphylactic: death in 6 minutes
639	Normal	4	582	21 days 1/100 cc. subcu- taneously	639	Anaphylactic: death in 7 minutes
704	16 days 1/1200 cc. subcu- taneously	6	725	14 days 1/100 cc. subcu- taneously	704	Anaphylactic: death in 4 minutes
697	Normal	6	723	14 days 1/100 cc. subcu- taneously	697	Anaphylactic: death in 4 minutes
571	21 days 1/100 cc. subcu- taneously	12	604	21 days 1/100 cc. subcu- taneously	571	Anaphylactic: death in 8 minutes
646	Normal	12	607	21 days 1/100 cc. subcu- taneously	646	Anaphylactic: lived
619	19 days 1/100 cc. subcu- taneously	Control	Intravenous injection of 0.025 cc. horse serum			Anaphylactic: death in 5 minutes

*Experiment II.* The pigs in this series designated as immunized had received a series of 6 injections of horse serum and upon the date of testing withstood the intravenous injection of 10 minimal anaphylactic doses without giving any evidence of anaphylactic symptoms.

The sensitized pigs which received the intracutaneous injections had been sensitized sixteen days previously with horse serum in amounts ranging from 1/1000 to 1/1500 cc. The sensitized pigs which received the tissue emulsions intravenously had all received a sensitizing injection of 1/100 cc. of horse serum fourteen days previously. Both lots of sensitized animals were susceptible to the intravenous injection of antigen upon the date of the test. The animals were tested by injecting 5, and 10 minimal anaphylactic doses but since the results with the former were less consistent than with the larger quantity, there are to be found in the table on page 276 only the results secured after the intracutaneous injection of 10 minimal anaphylactic doses. Antigen was permitted to remain in the tissues three and six hours.

It may be concluded from these data that, as in the preceding experiments, no distinction can be made between sensitized and normal pigs in respect to their ability to denature or eliminate antigen. In the case of the immunized pigs it is readily apparent that such pigs possess a mechanism which removes or alters antigen so that it can no longer be demonstrated as such after a few hours.

Several experiments similar to the foregoing were performed, in which passively sensitized pigs were substituted for the actively sensitized. The results paralleled those obtained with actively sensitized pigs in all essential respects. The only difference noted was that, as a rule, the results were more consistent, since passively sensitized pigs are subject to less variation in sensitization. These experiments as a whole, however, have a more direct bearing upon other work involving the specificity of antigenic elimination and consequently they will not be presented at this time.

While the anaphylactic experiment gave some indication of the differences in response of immunized, sensitized, and normal pigs to the intracutaneous injection of antigen it was not sufficiently delicate to detect slight variations. Therefore, the precipitin reaction, which presents fewer variable factors and is less subject to experimental error was proposed as a substitute for the anaphylactic test. To discover whether comparable re-

*Absorption test with 10 minimal anaphylactic doses of horse serum*

INTRACUTANEOUS IN- JECTION INTO PIG	TISSUE REMOVED AFTER	SENSI- TIZED PIG RE- CEIVING EMUL- SION	INTRA- VENOUS INJE- CTION OF 0.5 CC. TISSUE EMUL- SION FROM PIG	RESULT
	<i>hours</i>			
545 Immune	3	713	545	Anaphylactic: reinjected intra- venously 4 minimal anaphylac- tic doses after 4 days. Death 11 minutes
563 Immune	3	715	563	Anaphylactic: reinjected intra- venously 4 minimal anaphylac- tic doses after 4 days. Anaphy- lactic—lived
702 Sensitized	3	709	702	Anaphylactic: death in 5 minutes
703 Sensitized	3	712	703	Anaphylactic: death in 12 minutes
693 Normal	3	711	693	Anaphylactic: death in 5 minutes
694 Normal	3	710	694	Anaphylactic: death in 5 minutes
554 Immune	6	720	554	Anaphylactic: reinjected intra- venously 4 minimal anaphylac- tic doses after 4 days. Death in 6 minutes
556 Immune	6	719	556	Anaphylactic: reinjected intra- venously 4 minimal anaphylac- tic doses after 4 days. Death in 4 minutes
704 Sensitized	6	725	704	Anaphylactic: death in 4 minutes
705 Sensitized	6	726	705	Anaphylactic: death in 4 minutes
696 Normal	6	722	696	Anaphylactic: reinjected intra- venously 4 minimal anaphylac- tic doses after 4 days. No symptoms
697 Normal	6	723	697	Anaphylactic: death in 4 minutes
552 Immune	Control			Intravenously injected 10 mini- mal anaphylactic doses. No symptoms. Reinjected intra- venously 4 days later with 4 minimal anaphylactic doses. No symptoms
729 Sensitized	Control			Intravenously injected 0.05 cc. horse serum. Death in 4 min- utes

sults could be obtained with this procedure, an experiment was performed in which both methods were employed for detecting antigen in the same emulsions.

*Experiment III.* Pigs 972, 973, 974, and 975, were passively sensitized by the injection of 0.5 cc. of the serum of rabbit 24 (immunized to horse serum). Two days later 0.5 cc. of horse serum was injected intracutaneously. After twenty-four hours the tissue was removed and emulsions prepared. The content in horse serum was measured by both the anaphylactic and precipitin methods.

PIG	ANAPHYLACTIC TEST	PRECIPITIN TEST
972	Symptoms	Positive in 1 : 100
973	Symptoms	Positive in 1 : 100
974	Symptoms	Positive in 1 : 100
975	Severe symptoms	Positive in 1 : 250

Similarly, in pigs that had received subcutaneously a series of injections of horse serum, the reactions secured with tissue emulsions after the intracutaneous injections were comparable.

PIG	ANAPHYLACTIC TEST	PRECIPITIN TEST
983	Symptoms	Positive in 1 : 200
984	Symptoms	Positive in 1 : 200
986	Death in 4 minutes	Positive in 1 : 500 (higher values not tested)
989	Death in 8 minutes	Positive in 1 : 500 (higher values not tested)

The results indicate that the precipitin reaction not only runs parallel with the anaphylactic test but is also capable of furnishing a more exact measure of the residual antigen in the tissues. Therefore in the following work this method has been substituted for the anaphylactic procedure in the titration of emulsions derived from normal, sensitized, and immunized pigs.

*Experiment IV.* Pigs 999 and 1000 (sensitized), 1003 and 1004 (immunized), and 1009 (normal), were injected intracutaneously with 0.2 cc. of normal horse serum; killed twenty hours later; tissue emulsions prepared and tested for content in horse serum by titrating against

precipitating serum 127. The titer of serum 127 was at least 1 : 4000. The following values were obtained with tissue emulsions.

999	Sensitized	1 : 100
1000	Sensitized	1 : 200
1003	Immunized	1 : 100
1004	Immunized	1 : 25
1009	Normal	1 : 400

The fact that so large a quantity of extract was required in the emulsions from the immunized pigs to give a positive reaction with the precipitating serum indicates that a more rapid disappearance of antigen has occurred than in the normal pig, for example, where a positive reading is given in a dilution of 1 : 400. There is also a slight difference demonstrable between the sensitized and normal pigs. While these preliminary determinations were not carried out with the precision that would be required for quantitative work, since the titer of the precipitating serum was not determined exactly, the results were sufficiently favorable to warrant further investigation.

In order to eliminate individual variations which are present even though the animals are treated in an identical manner several intracutaneous injections were made in each of a series of sensitized, normal and immunized guinea-pigs. These injections were given in different parts of the skin and at different times. This, of course, raises the question as to whether the first injection may not modify the response to the others following. This point has not been decided. However, since all of the pigs, immunized, normal, and sensitized, received the same treatment this factor was neglected and the results of the treatment as a whole in one type of animal were compared with the results obtained in other types.

*Experiment V.* Pigs 1010, 1019 (normal); 1012, 1013 (sensitized); and 1015, 1016 (immunized), were injected intracutaneously as follows:—

December 17, 2.00 p.m., 0.2 cc. horse serum, right shoulder.

December 18, 8.00 a.m., 0.2 cc. horse serum, right hip.

December 18, 8.00 p.m., 0.2 cc. horse serum, left shoulder.

December 19, 8.00 a.m., 0.2 cc. horse serum, left hip.

December 19, at 8.10 a.m. these pigs were killed and the portions of injected tissue were removed. In addition, a portion of the tissue that had not been injected was removed from each pig. Emulsions were prepared by grinding and adding saline solution in amounts sufficient to give a 1 per cent suspension. The suspensions were cleared either by centrifugation or filtration, or both. Dilutions of these suspensions were made and used in the precipitin test with rabbit-antihorse serum. The several emulsions from each pig are designated by "a," "b," "c," and "d," corresponding with the intervals 42, 24, 12, and 0 hours. "e" represents the emulsion of the uninjected tissue. Results with duplicate pigs are given.

*Normal pigs*

EMULSION	POSITIVE IN	EMULSION	POSITIVE IN
1010 a	1 : 800	1019 a	1 : 800
b	1 : 800	b	1 : 600
c	1 : 2000	c	1 : 2000
d	1 : 4000	d	1 : 4000
e	Less than 1 : 50	e	Less than 1 : 50

*Sensitized pigs*

1012 a	1 : 100	1013 a	1 : 200
b	1 : 200	b	1 : 400
c	1 : 800	c	1 : 2000
d	1 : 4000	d	1 : 4000
e	Less than 1 : 50	e	Less than 1 : 50

*Immunized pigs*

1015 a	1 : 100	1016 a	1 : 100
b	1 : 200	b	1 : 100
c	1 : 400	c	1 : 800
d	1 : 4000	d	1 : 4000
e	1 : 50	e	1 : 50

This experiment was repeated, an identical procedure being employed. The results corresponded almost exactly with those given above.

Although the results of this work seem to indicate clearly that different rates of antigen absorption obtain, one should hesitate before drawing any broad generalized conclusions, since, but one antigen, horse serum, was employed. Nevertheless granting that we have been dealing with a special case, the results bring forth certain points which are suggestive.

The following curves (fig. 1) representing the values secured from an average of all animals of the respective types are based upon the percentages of residual antigen as expressed in the figures below:

	NORMAL	SENSITIZED	IMMUNIZED
a	12.5	4.5	2.5
b	19.0	9.0	4.5
c	50.0	36.0	11.0
d	100.0	100.0	100.0

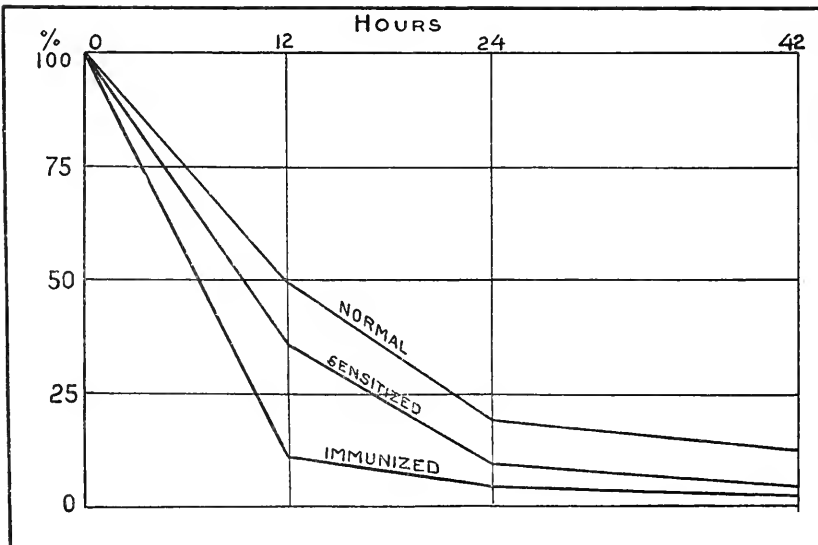


FIG. 1



Study of the curves shows the differences in reaction of the three types of animals. While there is an elimination of serum from all three types, the drop in the curve in the immunized pigs is much more abrupt than in the normal and sensitized. Between these last two there is much similarity, but the sensitized is throughout somewhat lower than the normal. A consideration of the time rate of the reactions shows that after twenty-four hours by far the larger per cent of serum is eliminated from the tissues of all three types. At the end of this period the differences in antigen content are only slight. It is of particular interest that the tissues of the immune pigs have eliminated very nearly as much antigen after twelve hours as the sensitized after twenty-four hours, and more than the normals after forty-two hours.

The character of the curves is the same for all three types of animals. Whatever differences there are would appear to be quantitative rather than qualitative. It is possible therefore to deduce from the curves the supposition that the processes of elimination of foreign protein from sensitized and immunized animals are simply exaggerations of processes normally present.



## THE INFLUENCE OF TYPHOID VACCINE ON TUBERCULOUS GUINEA-PIGS

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In the protection of large groups of men by typhoid vaccination, it has been customary to avoid giving the vaccine to those having active tuberculous lesions and often, also, to those with latent forms of tuberculosis. The reason for this has presumably been the dread of the effect of any febrile reaction or of any temporary lowering of resistance in the tuberculous patients and also the reported exacerbations of tuberculous lesions following attacks of typhoid fever. On account of the few cases of tuberculosis which have been injected with typhoid vaccine and because of the confusing complexity of conditions often found in clinical work, it has seemed of value to observe the effect of typhoid vaccination upon susceptible animals which had been inoculated with active tubercle bacilli.

The preliminary experiment of this series was made on two guinea-pigs of approximately the same age and weight. Each of these was injected on July 23, 1913, with 2 mgm. of human tubercle bacilli in suspension. One received no further treatment; the other was given an injection of typhoid vaccine (5,000,000 typhoid bacilli) on August 15, August 21 and August 29. The duration of life and the changes in weight of the two animals are shown in the accompanying charts. The animal injected with tubercle bacilli only, developed the usual course of the disease and was found at autopsy to have the usual lesions of tuberculous guinea-pigs. The animal which was injected with typhoid vaccine after the injection of tubercle bacilli took on an almost phenomenal increase in weight and was killed after four

and a half months seemingly in good condition (see Chart I, A and Chart I, B). The autopsy on this animal showed tuberculous changes, in the lungs and in the inguinal lymph node above the point of injection of the tubercle bacilli, which had developed extensive fibroid changes in an apparent effort to heal. But the spleen showed recent active miliary lesions of tuberculosis. None of the subsequent experiments showed so marked a difference in the nutrition of the vaccinated and the unvaccinated guinea pigs.

In the second experiment an overwhelming dose of tubercle bacilli was given to small guinea-pigs (6 mgm. of tubercle bacilli to animals weighing from 125 to 225 grams). The smaller animals succumbed rapidly to tuberculosis and in all the duration of life and the changes in weight were but little affected by the typhoid vaccine. The average length of life of the six guinea-pigs receiving typhoid vaccine after the tuberculous infection was seven days longer than the average length of life of the three control animals, which received the injection of tubercle bacilli only (see Chart II, A and Chart II, B).

In the third experiment 4 mgm. of tubercle bacilli were given to each of the guinea-pigs, which weighed from 214 to 305 grams at the beginning of the experiment. The four guinea-pigs receiving typhoid vaccine were each given *seven* injections of 5,000,000 typhoid bacilli. The two animals in this group which received tubercle bacilli only did not show any marked difference in their average weight from the four receiving also the typhoid vaccine. The average nutrition of those which were not given the vaccine was a little better than the average nutrition of the vaccinated guinea-pigs and the average length of life of those not vaccinated was fourteen days longer than that of the vaccinated animals. This experiment was the only one in which the unvaccinated guinea-pigs showed better nutrition and lived longer than those which received typhoid vaccination. It will be noted that in this experiment seven doses of vaccine were given extending over a period of nine weeks (see Chart III, A and Chart III, B).

These three preliminary experiments on small groups of guinea-

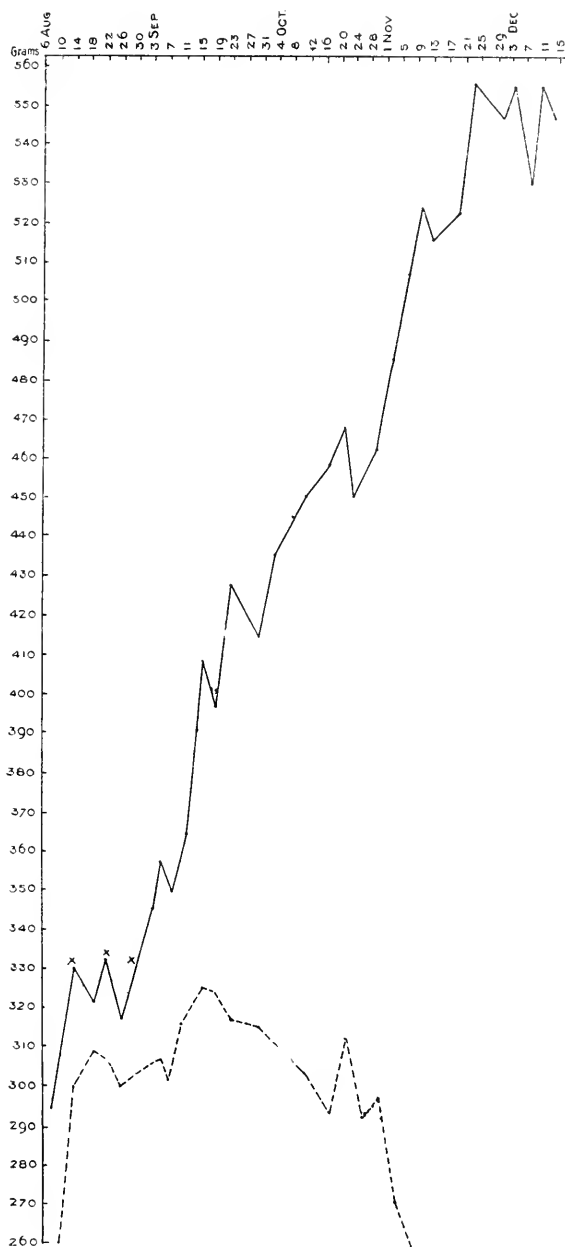


CHART 1A. WEIGHT CHART

July 23, 1913. Both guinea pigs received tubercle bacilli suspension. x Indicates date of injection with typhoid vaccine (5,000,000 typhoid bacilli).

pigs were made in the Herter Laboratory. The following observations which make up the major part of the work were

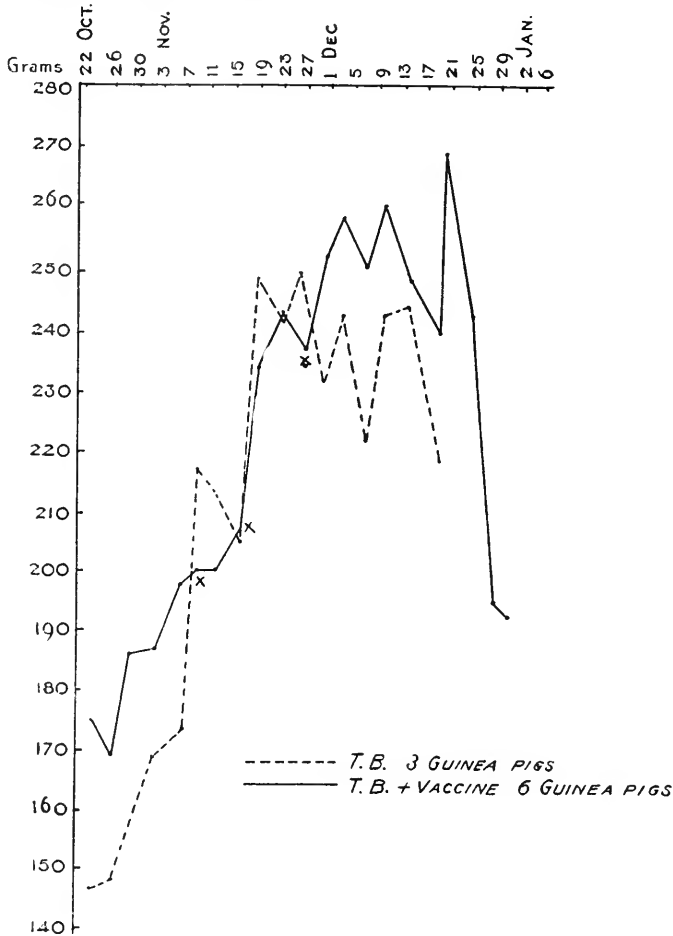


CHART II.A. AVERAGE WEIGHTS

On October 21, 1913, nine guinea pigs were injected with 6 mgm. tubercle bacilli suspension. x Indicates the date of injection with 5,000,000 typhoid bacilli (vaccine).

made in the Department of Pathology at Cornell University Medical College.

This later series, which was suggested by the results of the

early work at the Herter Laboratory was divided into 2 experiments:

1. To determine the effect of typhoid vaccine on large groups of tuberculous guinea-pigs with active lesions.
2. To determine the effect, if any, of typhoid immunization on the susceptibility of guinea-pigs to inoculation with virulent tubercle bacilli; i.e., immunizing the animals to typhoid and inoculating them later with tubercle suspension.

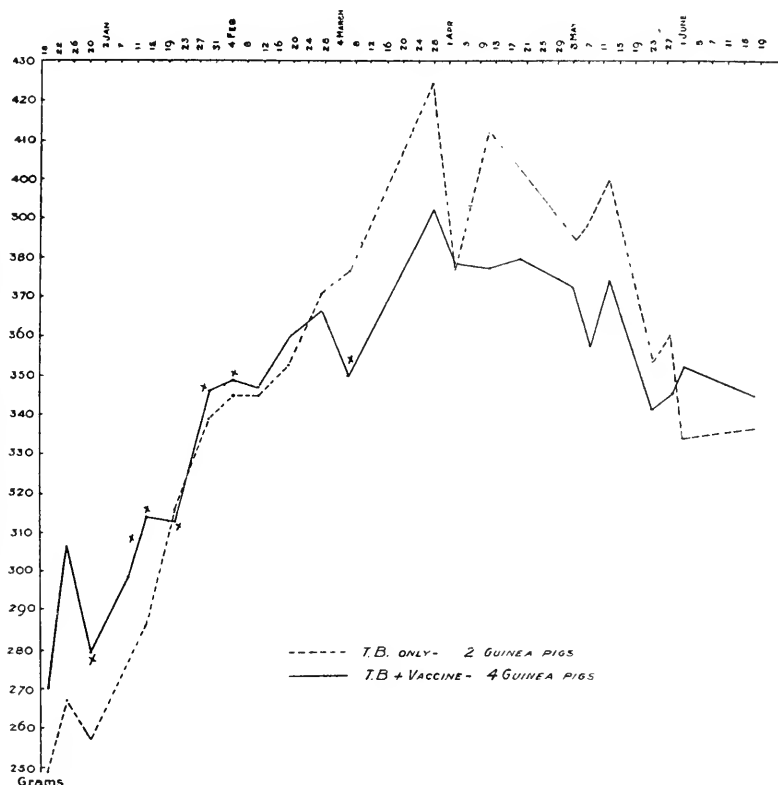


CHART IIIA. AVERAGE WEIGHTS

Injected December 18 with 4 mgm. tubercle bacilli in suspension. x Indicates injection of typhoid vaccine (5,000,000 bacilli).

In this work we endeavored to obtain by the use of larger groups of animals a more uniform average, in which individual

variation in nutrition and length of life would be a negligible factor.

Young healthy guinea-pigs of approximately equal weight were used. These animals were kept at least one week before inoculation to determine their normal condition; weights were taken on alternate days, an increase in weight indicating the health of the animals. The tubercle culture used was a virulent human strain in the second generation. A 2 mgm. suspension was made by grinding in a glass mortar and later diluted to required strength with normal saline and shaken vigorously for one hour. This suspension contained 2 mgm. of tubercle bacilli to 0.5 cc., which was adopted as the standard dose. The tubercle suspension for each series was prepared at the same time and injections were made on the same day into the animals that previously had received an immunizing dose of typhoid vaccine and also into the unimmunized animals.

The typhoid vaccine employed was that made by the New York City Board of Health and in general use for typhoid immunization. In the first series carried on at the Herter Laboratory the dose of vaccine was regulated according to the weight of each guinea-pig. Further observation, however, demonstrated that the larger doses (the adult human dose, 500,000,000 to 1,000,000,000) did not produce detrimental effects on normal guinea-pigs and this was therefore adopted as the dose in the present series of experiments. The typhoid vaccine inoculations in each series were made at the same time and with the same preparation of vaccine. Daily observations were made on these animals and their weights were taken on alternate days during the first weeks twice weekly for three months and at intervals during one year. Autopsies were performed on all the animals and cultures were made from the spleen and mesenteric lymph nodes from several guinea pigs in each series, first, to recover the tubercle bacilli and secondly, to exclude the presence of mixed infection.

The fourth experiment included twenty-one healthy young guinea-pigs of approximately equal weight which were injected with a 2 mgm. standard suspension of tubercle bacilli on October



1913 Killed  
Dec. 12  
P

Dec. 11  
Dec. 1  
Nov. 30  
Nov. 20  
Nov. 10  
Oct. 31  
Oct. 21  
Oct. 11  
Oct. 1  
Sept. 21  
Sept. 11  
Sept. 1  
Aug. 22  
Aug. 12  
Aug. 2  
July 23

1914  
Jan. 9  
Dec. 30  
Dec. 20  
Dec. 10  
Nov. 30  
Nov. 20  
Nov. 10  
Oct. 31  
Oct. 21

July 26  
July 16  
July 6  
June 26  
June 16  
June 6  
May 27  
May 17  
May 7  
Apr. 27  
Apr. 17  
Apr. 7  
Mar. 28  
Mar. 18  
Mar. 8  
Feb. 26  
Feb. 16  
Feb. 6  
Jan. 27  
Jan. 17  
Jan. 7  
Dec. 26  
Dec. 19

CHART IB

Length of life: T. B.  
only, 108 days; T. B.  $\pm$   
Vaccine 142 days; -----  
T. B. only; ——— Vaccine  
 $\pm$  T.B.

CHART IIB

Length of life: T. B.  
only, average 35.3 days;  
T. B.  $\pm$  Vaccine, 42.3  
days.

CHART IIIB

Length of life: T. B.  
only, 184 days; T. B.  $\pm$   
Vaccine, 70 days.

24, 1914. Thirty days after this tubercle injection, fifteen of these animals received the first dose of typhoid vaccine (500,000,000). The second dose of vaccine (1,000,000,000) followed in eight days and the third dose eight days later. The remaining six pigs were not vaccinated. The average weight curve of the animals receiving the vaccine shows an early increase in weight with sudden loss at the end of about three weeks. This loss was later regained and appeared to be unaffected by the typhoid injection. Of the control animals, that is, the six guinea-pigs receiving only the tubercle suspension; three were injected on October 24. Of this group two died within thirty-three days, so that the greater part of the control weight curve in Chart IV represents the life of one guinea-pig and not an average. Three other guinea-pigs were injected with tubercle bacillus suspension on December 18 in order if possible to procure a fairer average for the controls. This second series of controls is represented in Chart IV by the double dotted line.

It must be noted that these animals were heavier at the time of inoculation than any of the other 18 guinea-pigs, so that the average curve at first shows an almost stationary weight, later a definite loss in weight (see Chart IV, A). The average length of life of the animals treated was twenty-six and three-tenths days longer than the untreated, as is shown in Chart IV, B.

On autopsy the animals treated with vaccine in the majority of cases presented a peculiar atypical fibrous tubercle, which, in some instances, was localized in the liver, associated with early miliary tubercles in other organs. In other cases the organs showed many smaller foci, but rarely an acute lesion. In fact, in this group only one of the treated animals failed to show this fibrous or healing type of tubercle.

The control animals, on the other hand, developed a typical acute tuberculosis with extensive involvement of all the organs, caseation and necrosis being a prominent feature.

In the fifth experiment we endeavored to determine the effect, if any, of typhoid immunization on the susceptibility of guinea-pigs to tuberculous infection. Of nineteen healthy guinea-pigs ten were selected and given three immunizing doses of

typhoid vaccine at weekly intervals. Eight days after the last typhoid injection all the nineteen guinea-pigs received a 2 mgm. standard dose of tubercle bacillus suspension. After an interval of thirty-three days five of the animals that had not received the immunizing doses of vaccine were injected with typhoid vaccine as in the preceding experiment.

Those animals receiving the typhoid vaccine before the tubercle inoculation showed an early gain in weight which was later followed by a distinct loss in general nutrition but later

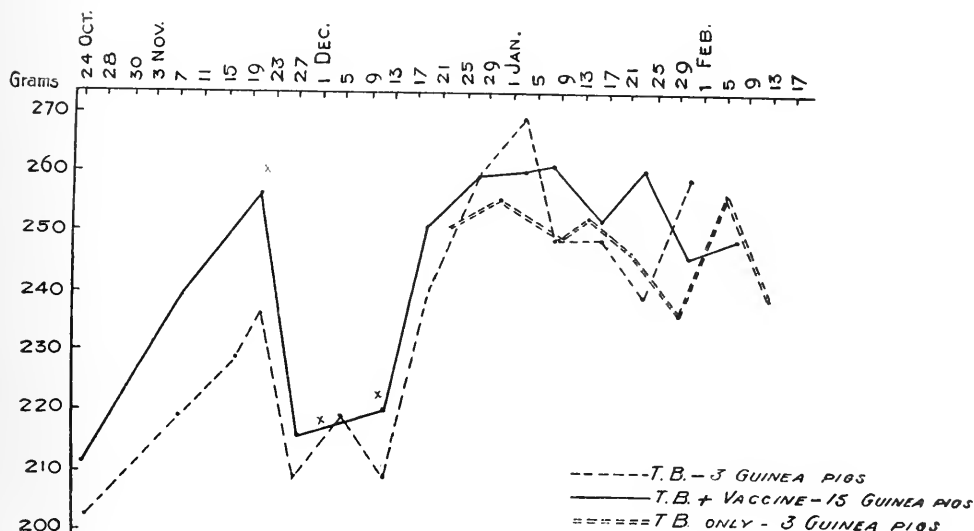


CHART IVA. AVERAGE WEIGHTS OF TWENTY-ONE GUINEA PIGS

Injected October 24, 1914, with 2 mgm. tubercle bacilli in suspension. x Indicates date of injection with typhoid vaccine.

this loss was regained and ultimately the nutrition in both groups of animals treated with typhoid vaccine appeared about equal. (Chart V, A).

The four remaining animals receiving the tubercle suspension only, after wide variations in loss and gain reached a nutritional average far below that of the other two groups (Chart V, A). These untreated animals developed a very active tuberculous lesion and three died within six weeks.

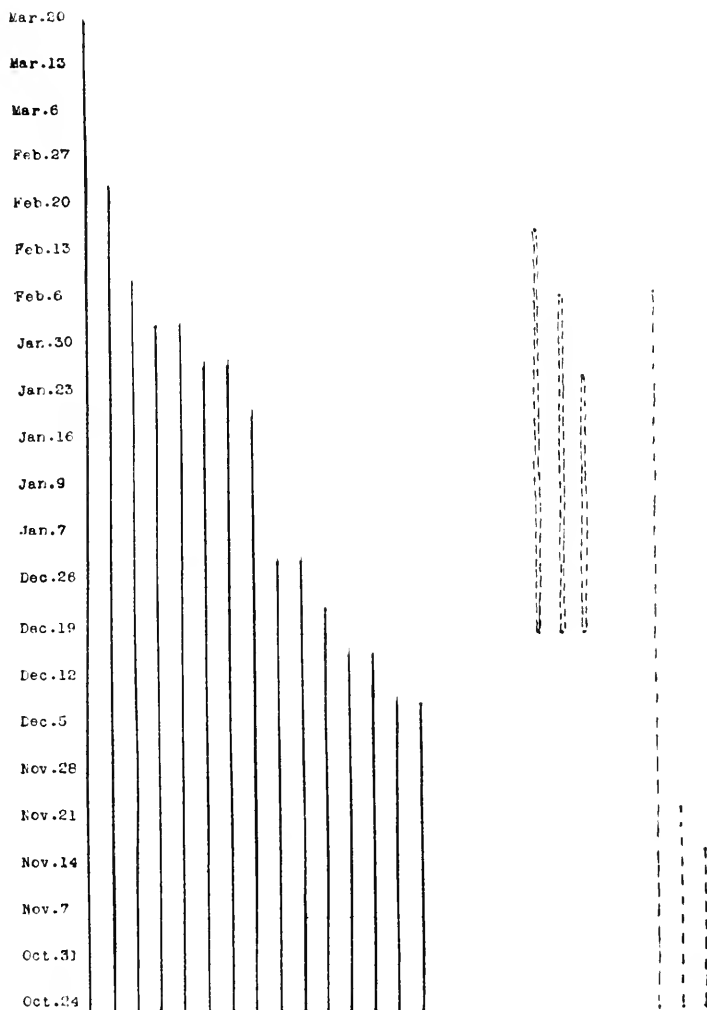


CHART IVB

Showing length of life of guinea pigs inoculated with tubercle bacillus suspension and tubercle suspension and later inoculated with typhoid vaccine. ——— T. B. + Vaccine; ----- T. B. only; ===== T. B. only<sub>2</sub> (later injection).

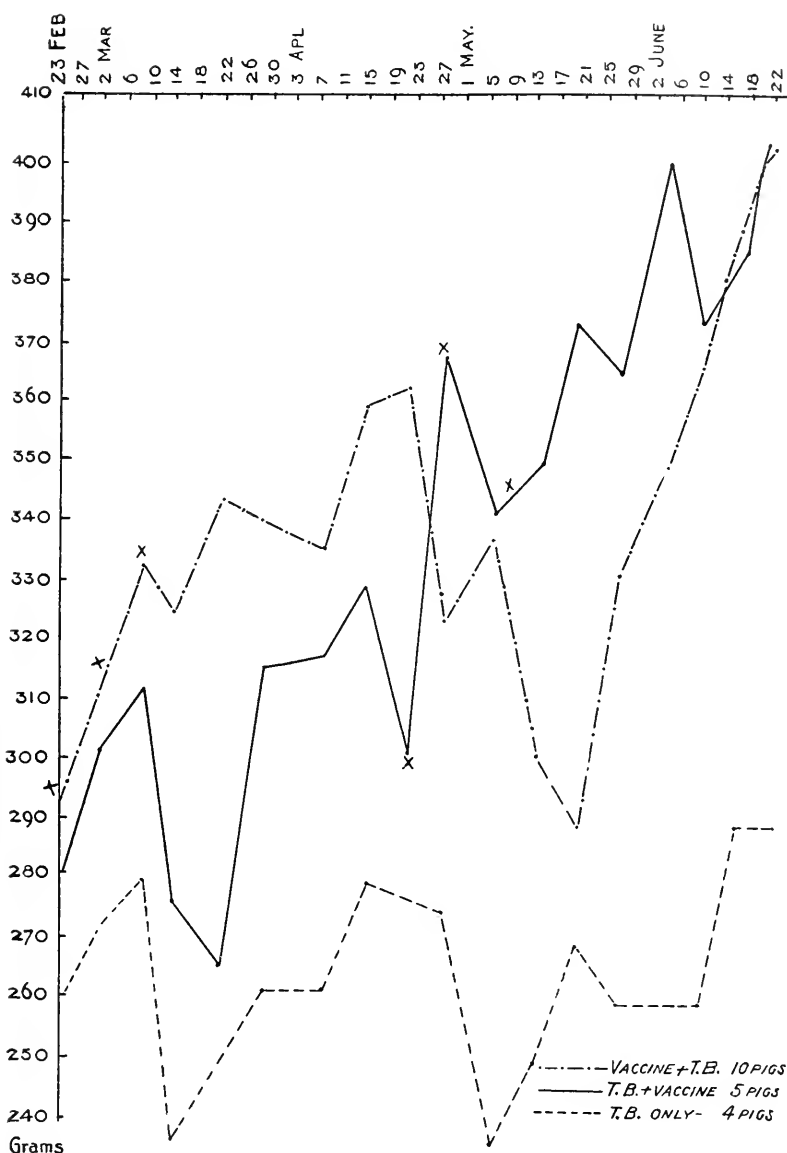


CHART VA. AVERAGE WEIGHTS OF TWENTY GUINEA PIGS

Ten animals received immunizing doses of typhoid vaccine and later were inoculated with tubercle bacilli. Five were given tubercle bacillus suspension and later treated with typhoid vaccine. Four received tubercle bacilli suspension only. x Indicates date of typhoid vaccine.

The average length of life of the three groups in this series are shown in Chart V, B. The nutrition appears slightly better and length of life somewhat longer in those animals receiving the typhoid vaccine after the tubercle inoculation than in those previously immunized. In the former group the average length of life was 89.6 days longer than in the latter group.

The untreated group is far below the average of the treated group both in nutrition and apparent resistance, the animals treated with vaccine having an average length of life 127.1 days longer than the untreated.

It is especially to be noted that in this group two of the guinea-pigs treated with typhoid vaccine were killed one year after inoculation and the autopsy showed in one instance firm fibrous masses in the liver with small tubercles scattered through both lungs. In another case the only lesion was an old hyperplastic tuberculous pericarditis with fibrous tuberculosis in inguinal lymph nodes. In the third animal the only active lesion was fine miliary tubercles in both lungs with only fibrous tuberculous lymph nodes.

In the sixth experiment considerable difficulty was encountered in obtaining healthy guinea-pigs. Twenty out of the fifty animals received for the experiment died within the first week of observation before any treatment had been started, apparently of an acute pulmonary congestion. The remaining thirty animals appeared lacking in resistance. The nutrition of the three groups appears about equal during the earlier weeks. The groups receiving the typhoid vaccine after injection with tubercle suspension perhaps develop a slight nutritional advantage later.

The autopsies on this group of vaccinated animals also showed a higher percentage of active lesions, especially of the acute miliary type, than was observed in any of the other previous experiments. The interesting feature in this experiment is the fact that given a group of apparently non-resistant animals neither the immunization with typhoid vaccine nor the later injection of this vaccine appears to have any effect on the development or progress of the tuberculous process.

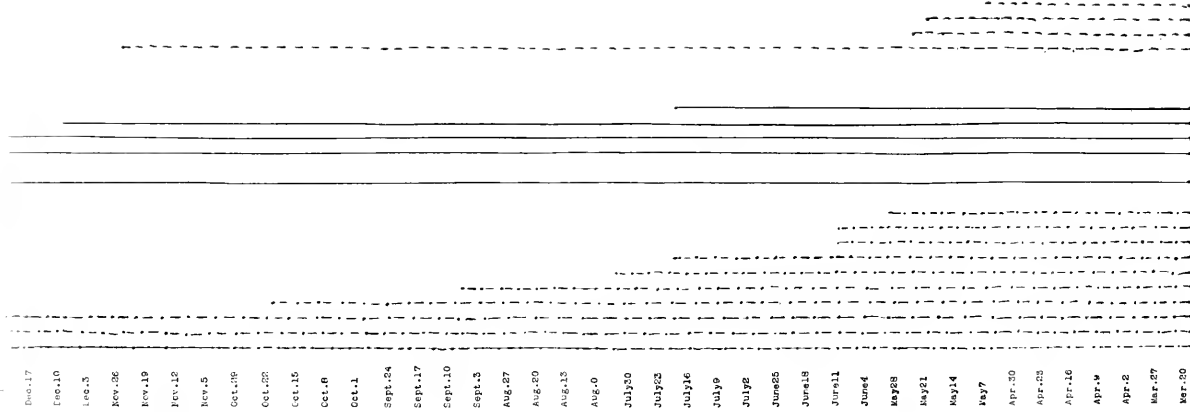


CHART VB

Showing length of life of 4 animals injected with tubercle emulsion and<sup>5</sup> inoculated with tubercle emulsion and later treated with typhoid vaccine and 10 treated with typhoid vaccine and later receiving the standard dose of tubercle emulsion. Average for those receiving tubercle bacilli only is 101.7 days. Those receiving immunizing doses of typhoid vaccine 184 days and those treated with typhoid vaccine after the tubercle inoculation 273.6 days.





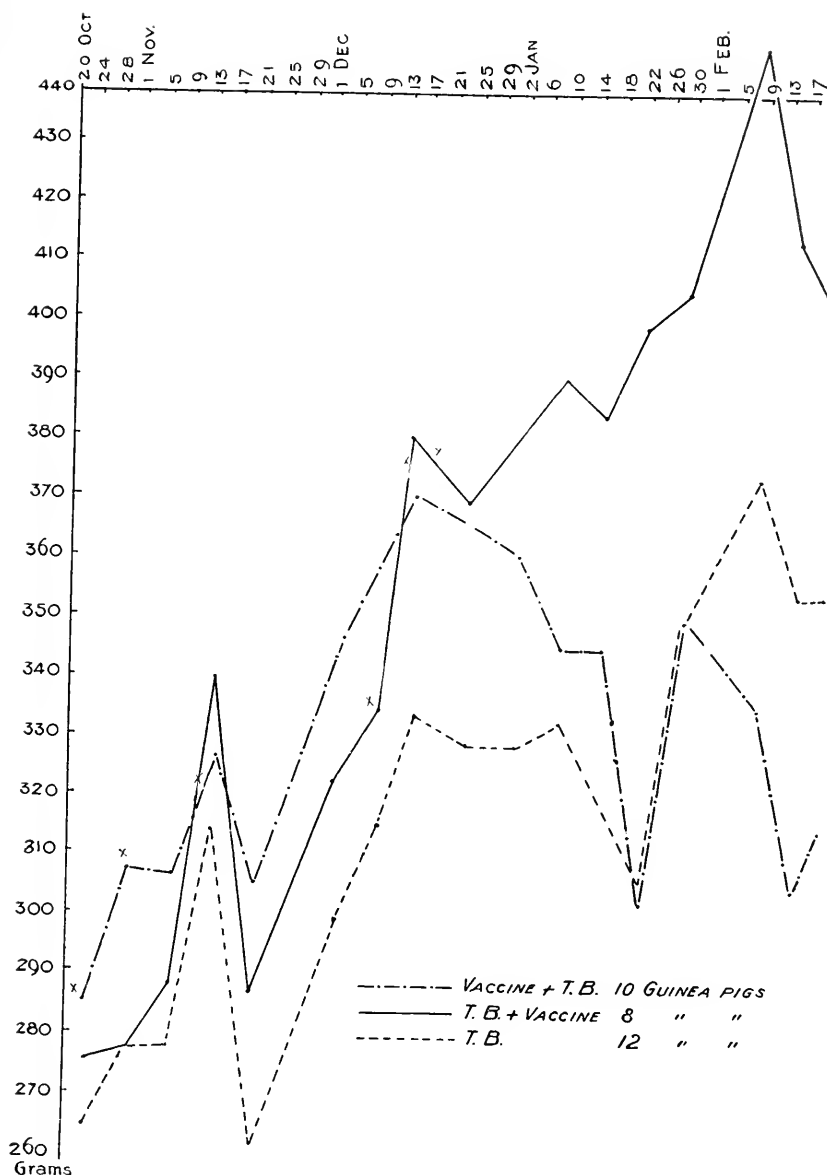


CHART VIA. AVERAGE WEIGHTS OF THIRTY GUINEA PIGS

November 18, 1915, all injected with a 2 mgm. tubercle bacilli suspension.  
 x Indicates the injection of typhoid vaccine adult human dose 500,000,000 to 1,000,000,000.

## DISCUSSION

1. *Duration of life.* The length of life of the guinea-pigs varied greatly, both of those receiving tubercle bacilli only and of those also injected with typhoid vaccine. Taking a

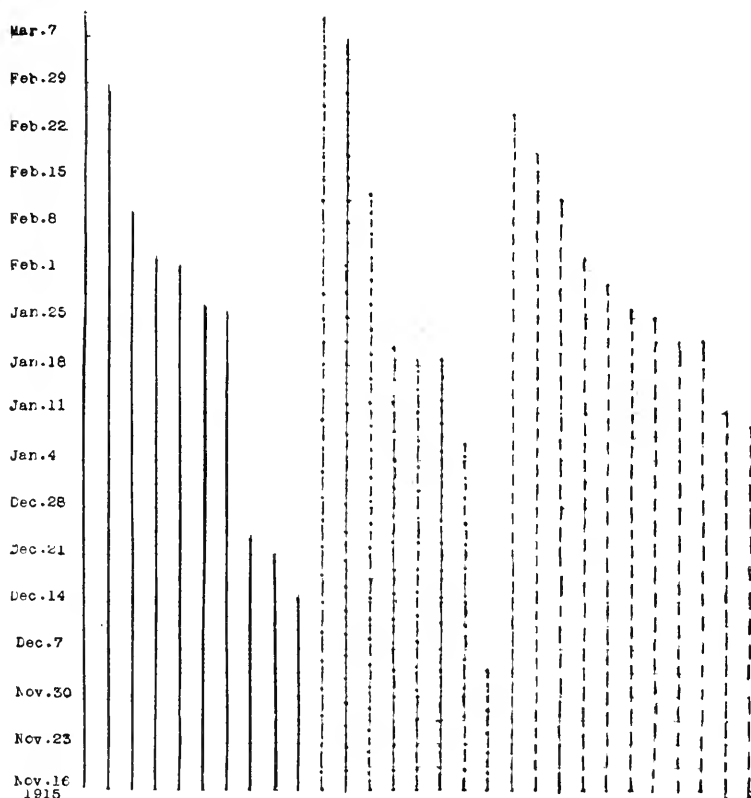


CHART VIB. DURATION OF LIFE OF VACCINATED AND NON-VACCINATED GUINEA PIGS

Animals receiving tubercle suspension of tubercle bacilli plus typhoid vaccine show an average of 67.5 days. Those receiving typhoid vaccine and later inoculated with tubercle bacilli average 67 days. Tubercle bacilli only average 66 days. — = T. B. + Vaccine; --- = Vaccine + T. B.; ..... T. B. only.

general average of all the animals under observation, that is, of twenty-eight inoculated with tubercle bacilli which received no other treatment and sixty animals injected with typhoid vaccine

in addition to the tuberculous infection, the average duration of life of those vaccinated was thirty-two days longer than of those not vaccinated. This was in spite of the fact that three of the vaccinated animals were killed after having long outlived their controls and when in an apparently excellent condition.

When the animals were given an overwhelming dose of tubercle bacilli (6 mgm.) the vaccine had almost no influence on the length of life, the vaccinated animals living on an average seven days longer than the ones not given vaccine.

In the small group of animals given seven doses of vaccine during a period of nine weeks, the vaccinated animals lived on an average fourteen days less than the controls but there were only four of these guinea-pigs, too few to draw any conclusions from them.

2. *Nutrition.* The guinea-pigs under observation were all young growing animals and all gained weight for a period after the injection of the tubercle bacilli. The weight charts of the animals showed marked variations both in the cases of those that were given vaccine and those not vaccinated. Some of the tuberculous guinea-pigs after receiving the typhoid vaccine showed a temporary loss of weight but this loss of weight was in almost all the cases later regained and in a number of the animals the weight increased very rapidly and very markedly after the vaccination. Some of the guinea pigs that took on this almost phenomenal increase in weight after the vaccination continued for weeks or months in this markedly good condition although still having an active tuberculous process.

3. *Lesions.* The autopsy findings are of interest in that those animals receiving the typhoid injections in the majority of cases showed changes resembling fibrous tuberculosis, suggesting a healing process. This is taken to indicate an increased resistance as compared with the control animals which demonstrated the usual type of active tuberculosis, namely necrosis and extensive caseation with relatively little attempt at fibrosis.

In one group both the vaccinated and non-vaccinated animals showed at autopsy lesions resembling acute miliary tuberculosis. These animals lived a relatively short time and were unable

to develop any resistance. Even in this group, however, the lesions in the vaccinated animals appeared less active and extensive than in the non-vaccinated.

4. *General effect of typhoid vaccination in tuberculous guinea-pigs.* In this group of sixty guinea-pigs which received injections of typhoid vaccine after being inoculated with tubercle bacilli, it is shown that while there was in some instances a temporary loss of weight after the vaccination, there was no other apparent ill-effect following the use of the vaccine.

On the other hand these experiments seem to indicate that typhoid vaccination not only does not lower the resistance of tuberculous guinea-pigs but may render them more resistant and to suggest the beneficial effect of an excess of foreign bacterial protein. However, since apparently healthy persons may have a definite increase in weight following typhoid vaccination, it may be that the increase in weight and the greater resistance to the tuberculous infection shown in these guinea-pigs was due to a stimulation of the nutrition by the vaccine in some way which is not primarily anti-bacterial.

The recent experiments of Jobling (1) and the favorable results obtained by injection of foreign proteins would favor the former theory. We have further studies in progress to determine the effect of other organisms than the typhoid bacillus in tuberculous animals and also to observe the effect of injection of non-bacterial foreign protein in this group of cases.

The injection of alien bacterial vaccines into individuals already the subject of active infection with another organism has from time to time been suggested. Von Dungern in a series of experiments demonstrated beneficial effects, in cases of anthrax following the injection of large doses of killed Friedlander bacilli. More recently Kraus (2) has shown marked improvement in cases of typhoid following injection of large doses of colon bacilli and typhoid vaccine gave marked benefit in certain pelvic infections. Whether it is permissible to transfer the results obtained by animal experimentation to human conditions is a question. The fact remains, however, contrary to the generally accepted idea; first, that in this series of animals

there appeared to develop a greater resistance to tubercle inoculation in the immunized than in the unimmunized animals, and secondly, that animals with active tuberculosis when given typhoid vaccine appeared to have an increased resistance.

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# COMPLEMENT FIXATION IN TUBERCULOSIS WITH THE "PARTIAL ANTIGENS" OF DEYKE AND MUCH

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Since the fundamental observations of Wassermann and Bruck (1), and later of Citron (2), on the occurrence of tuberculous antibodies in the extracts of organs and tissues of tuberculous patients, which antibodies are demonstrable by complement fixation, many attempts have been made by workers in tuberculosis to develop a complement fixation test in tuberculosis of a diagnostic value comparable to that of the Wassermann reaction in syphilis.

This work has passed through many phases and has, in the main, given rise to inconstant results, the results varying with the antigens used by various workers. The literature on this subject has been recently abstracted by H. R. Miller (3) and the various antigens used by the several workers tabulated in four general groups. *Group I.* This group comprises those antigens composed of the whole bacillus, including not only antigens prepared from the tubercle bacilli, but also antigens prepared from other allied acid-fast bacteria. The results reported vary both in constancy and the percentage of positive fixation in positive cases. *Group II* are antigens which consist of various tuberculins. In the main the results reported by these workers have been encouraging, the chief difficulty being non-specific fixation, especially with the sera of non-tuberculous syphilitic patients. *Group III* are those antigens derived from the tubercle bacillus by means of chemical digestion and extraction. The chief workers in this field have been Deyke, Much,

Leschke and Altstaedt. It is to this group that the "partial antigens" belong. *Group IV* comprises antigens derived from normal and tuberculous tissue. Such antigens have not given uniform results.

While the attention of the majority of workers in tuberculosis has been directed to tuberculin or whole bacillus antigens, little attention has been given in this country to the question of partial antigens of the tubercle bacillus. The fundamental observations and theories of Deyke and Much upon the partial antigens and their relationship to the treatment in tuberculosis, offer a distinctly different viewpoint.

#### HISTORICAL<sup>1</sup>

In 1908 Deyke and Much (4, 5) observed that tubercle bacilli in an emulsion of brain tissue rapidly lost their acid fast properties. Believing that this was due to the action of neurin or cholin, dried tubercle bacilli were subjected to their action. A marked disintegration of the bacilli resulted. The emulsion of disintegrated bacilli, called by the authors "Tb-L," was toxic to guinea-pigs. Believing this toxicity might be due to the endo-protein, the protein was extracted by subjecting the bacilli to alkylamine, filtering, and extracting the residue with acetic acid. The resulting product, called "Tb-A," was believed to be the pure protein of the bacilli.

Much (6), influenced by Deyke's work on the neutral fat of leprosy bacilli, isolated a similar fat body—"tuberculo-nastin"—from the tubercle bacillus. This tuberculo-nastin as an antigen gave fixation of complement with tuberculous sera. Vaccination experiments led him to believe this tuberculo-nastin was capable of evoking specific antibodies.

In 1909 Deyke and Much (7) reported upon the relationship of these components of the tubercle bacilli to immunity in tuberculosis. Guinea-pigs were immunized respectively to old tuberculin, tuberculo-nastin, Tb-L, and Tb-A, and to a combination of nastin and Tb-A (N-Tb-A). These pigs were subsequently given intraperitoneal injections of living tubercle bacilli. It was found that old tuberculin, nastin, and Tb-A, by themselves, conferred no immunity. However,

<sup>1</sup> Owing to the length of this article, a review of the work of Besredka, Bronfenbrenner, Craig, Petroff, and Miller and Zinsser has been omitted.



the animals immunized to Tb-L (containing all the elements of the bacillus) and to N-Tb-A, showed a decided immunity to the subsequent infection. The authors therefore concluded that the immunity to tuberculosis depended upon an immunity to all the factors of the bacillus. The therapeutic possibilities of this observation were pointed out—the possibility of determining by serological methods the factors of the tubercle bacilli to which an individual was lacking in specific antibodies, and of accordingly supplying these by vaccination, or active immunization, with the appropriate partial antigen. This idea is later elaborated by Much (8). In 1911, Much and Leschke (9) determined, by serological studies in guinea-pigs immunized to the partial antigens of various acid fast bacteria, the relationship between the component parts of the tubercle bacilli and the similar components of other acid fast organisms. While the several sera of animals immunized to the various partial antigens of the tubercle bacillus showed a slight variable fixation with non-specific partial antigens, with partial antigens of the tubercle bacillus complete and constant fixation of complement resulted. They concluded therefore that the partial antigens of the tubercle bacillus were specific in their immunological reactions. With the collaboration of Dielman, this same specificity was shown by complement fixation with human tuberculous sera. Further methods for the splitting up of the tubercle bacillus were presented at this time. These methods were:

1. *Benzoyl chloride method.* Ten grams of washed living tubercle bacilli were subjected to the action of 90 cc. of benzoyl chloride, for twenty-four hours with frequent shaking. The mixture was then filtered, the residue representing tuberculo-albumen. The filtrate was extracted with alcohol and then with ether, the ether extraction yielding tuberculo-nastin.

2. *Potassium hydroxide method.* Washed tubercle bacilli were emulsified in 2 per cent KOH. After standing the emulsion was neutralized with 2 per cent HCl, and then filtered. The residue was washed and then extracted with alcohol. The filtrate from the alcohol extraction represented the fatty-acid-lipoids of the bacilli.

Experimentally, tuberculo-nastin gave the strongest complement fixation with tuberculous sera and the tuberculo-albumen gave the weakest reaction. Of 77 human sera giving complement fixation with a whole bacillus antigen, 56 gave fixation with nastin, 14 with the fatty-acid-lipoid antigen, and but 7 with tuberculo-albumen. Tuberculo-nastin, however, could not produce specific antibodies when injected

into animals by itself. It was only when in a happy mixture with other antigens that it called forth antibodies.

Leschke (10), in the same journal, reviews the methods for obtaining partial antigens from the tubercle bacillus, and gives the details of the method using organic acids, a method previously mentioned by Much (11). (The details of this method are given under "Technique.")

In 1913, Deyke and Much (12), reported further investigations with these partial antigens. The lactic acid method was found to be the method of choice. As a result of their own and other investigations with contemporaneous complement fixation and vaccination tests with these partial antigens in both normal and tuberculous individuals they here state their idea of the nature of immunity in tuberculosis—a view stated previously by Much (13).

They regard the immunity in tuberculosis as two-fold—humoral and cellular, a humoral immunity which is manifested by the presence of partial antibodies in the blood serum and a cellular immunity as evidenced by positive vaccination to the same partial antigens. All individuals, except those in the terminal stages of tuberculosis and those in the first year of life, show a cellular immunity to tuberculosis. Humoral immunity they found present in early cases of tuberculosis, in active cases (except those in the terminal stages of tuberculosis), in children, and in adults that had recently overcome, or been continuously exposed to tuberculosis. The authors believed, therefore, that these two types of immunity were not parallel. In an organism actively fighting tuberculosis, humoral antibodies are needed for a successful fight. But in an organism not needing to fight an active infection, but at the same time prepared for defense, only the cellular immunity is present. In other words, cellular antibodies mean a preparedness for defense against infection, humoral antibodies mean the presence of weapons of offense against a present, or recently overcome infection. With the termination of a successful fight against a tuberculous infection, the humoral immunity disappears, while the cellular immunity remains. In other words, the presence of partial antibodies in the serum denotes either an active fight against tuberculosis, an active existing tuberculosis, or a very recently overcome infection.

Altstaedt (14) took up this work from both the diagnostic and therapeutic standpoints and his results largely confirm the findings and theories of Deyke and Much. In his first communication he emphasizes the precautions to be observed in complement fixation and the

technique necessary for successful results with these antigens. As this technique is practically identical with that followed by us, it is unnecessary to go into the details at this point (see below "Technique").

Altstaedt found by complement fixation with these partial antigens that children under one year of age showed no humoral partial antibodies, while children between the ages of one year and fifteen years showed large quantities of the humoral partial antibodies, indicating a recently overcome infection. Incipient and well advanced cases of tuberculosis showed humoral partial antibodies, only those cases in the terminal stages of their disease lacking them. Clinically healthy adults showed no humoral partial antibodies, with the exception that these bodies might at times be present in individuals exposed to contact with tuberculosis over a long period of time. Furthermore, Altstaedt showed that partial antibodies could be evoked by vaccination with the specific partial antigen, in those individuals lacking the same.

With elaborate vaccination tests, the technique of which he gives at length, Altstaedt found that positive results were obtained in practically all cases except those in the first year of life and those in the terminal stages of their disease. The strength of the cutaneous reaction was increased only slightly by repeated vaccination, although by this same means, the humoral antibody content increased decidedly.

In his latest communication, Altstaedt (15) gives the results of therapy by vaccinating tuberculous patients with those antigens in which complement fixation had shown they were lacking. In a period covering two and a half years, 297 patients were treated by this means. In many cases Altstaedt reports brilliant clinical results, in other cases the results seemed decidedly beneficial, while in no case was any apparent harm done the patient by the vaccination.<sup>2</sup>

With the idea of applying these methods to the study of tuberculosis, we have attempted to repeat some of the work of Deyke and Much, especially those phases which apply particularly to the diagnosis of the disease. We have borne constantly

<sup>2</sup> Von Ruck (16), in his work upon experimental immunization against tuberculosis, prepared "partial" antigens after the technique of Deyke and Much, and utilized these antigens to follow the degree of immunization in the experimental animals. Von Ruck emphasizes the fact that these partial antigens as prepared by him were not pure antigens in the chemical sense of the word but all contained traces of protein, and represented merely rough biological groupings of the various constituents of the tubercle bacilli.

in mind the possibility of ultimately using these partial antigens in the therapy of tuberculosis, should our preliminary work prove of sufficient value to warrant such a step.

In preliminary work we prepared, or endeavored to prepare, the several partial antigens of the tubercle bacillus by each of the three principal methods elaborated by Deyke and Much and their co-workers; namely, the benzoyl chloride method, the potassium hydroxide method, and the lactic acid method. With the first two methods, the benzoyl chloride and the potassium hydroxide methods, we had no success. The "partial antigens" resulting from both these processes were all either totally lacking in antigenic properties or anti-complementary. Our failure to obtain suitable antigens by the use of these methods, we believe, is due to the fact that either we did not follow the proper quantitative relationships between the disintegrated bacilli and the solvents, or that some step has been omitted in the directions given by the original authors. The papers of Deyke and Much presenting these methods are somewhat unsatisfactory in that few of the quantitative relationships and details are given. This probably explains our failure to obtain suitable antigens by either of these methods. The antigens resulting from lactic acid digestion were, however, suitable for use, with the exceptions noted under "Technique."

Our failure to obtain suitable antigens from either the benzoyl chloride or the potassium hydroxide method, induced us to abandon these two methods of preparing partial antigens, and to confine our efforts to the lactic acid method, which is stated by Deyke and Much to be the method of choice, and in our hands, has, in the main, yielded good results.

## TECHNIQUE

### *1. Preparation of antigens*

A strain of human tubercle bacilli, called the "Korn Culture" was used in the preparation of antigens. This strain was somewhat attenuated, and grew readily over the surface of Dorset's glycerine broth. The bacilli were removed by filtration and centrifugalization, and well washed with sterile distilled water.

Moist, well washed tubercle bacilli were added up to a 5 per cent dilution, to a 1 per cent lactic acid solution. This mixture was digested with shaking at irregular intervals for five weeks at a temperature of 58°. This lactic acid emulsion became tinged a faint yellow, gradually deepening to a brown, as the digestion proceeded. The degree of disintegration of the bacilli could be followed by microscopic examination. At the end of five weeks the emulsion was filtered. The filtrate (which was called Filtrate A) representing the water soluble fraction, is said by Deyke and Much to contain very small amounts of dissolved albumin, a minute quantity of fats, and to closely resemble tuberculin in its action. Deyke and Much attempted to purify and extract further this water soluble fraction and state that the addition of alcohol produced a precipitate. With us no precipitate resulted on the addition of alcohol, and we were unsuccessful in our efforts further to extract the water soluble fraction. In explanation of this it may be said that the original authors state that it is but occasionally that suitable antigens were obtained from the water soluble fraction.

The precipitate resulting from the filtration of the original lactic acid emulsion, containing the bulk of the protein and fats of the tubercle bacilli was taken up in 100 cc. of absolute ethyl alcohol, and extracted at 0° in the ice box for twenty-four hours. At the end of that time it was filtered, a clear filtrate resulting, which did not settle out upon warming to room temperature. This filtrate we called "Filtrate A.E." and represented the first alcohol extraction at 0°.

The precipitate remaining from this filtration was again taken up in 100 cc. of absolute ethyl alcohol and extracted at 37° for twenty-four hours and then filtered. This filtrate was labeled "Filtrate A.F." Upon cooling a light flocculent precipitate settled out, which was not disturbed.

The residue remaining from this extraction was again taken up in 100 cc. of absolute ethyl alcohol, and extracted at 56° for twenty-four hours, and then filtered. This filtrate was labeled "Filtrate A.G." Upon cooling, a light flocculent precipitate settled out. The residue from this extraction was further taken up in 100 cc. of absolute ethyl alcohol and extracted twenty-four hours at 78°, alcohol being added from time to time to make up for loss from evaporation. At the end of twenty-four hours this was again filtered, a clear filtrate resulting, in which a light flocculent precipitate settled out upon cooling. This filtrate was labeled "Filtrate A.H." and represents the fourth alcohol extraction of the original precipitate.

The residue remaining from this extraction was then taken up in 100 cc. ether and extracted at room temperature for twenty-four hours, and then filtered. The filtrate was labeled "Filtrate A.I." and probably represents chiefly neutral fats. In this filtrate also a slight precipitate gradually settled out.

The residue from this last ether extraction was then taken up in 100 cc. salt solution and labeled "Tuberculo-Albumen."

We had then from this method the following partial antigens. "Filtrate A, A.E., A.F., A.G., A.H., A.I.," and "Tuberculo-Albumen," representing supposedly the tuberculin, the various fatty acids and lipoids, the neutral fats, and the tuberculo-albumen. All of these partial antigens, however, gave a positive Tanret's and Biuret reaction.

#### *Titration of antigens*

Owing to the fact that we had no known positive or known negative sera, the various sera against which the antigens were titrated were taken from individuals differing in their status to tuberculosis as widely as possible, ranging from patients in the last stages of tuberculosis, to normal persons. The antigens were titrated in the usual manner, and an antigen was considered valuable if it gave complete inhibition of hemolysis with a serum from a tuberculous individual, in a dilution twice that in which it also showed complete hemolysis with any other human sera.

Of the antigens prepared by the lactic acid method, only two proved to be without value, namely, Filtrate A, supposedly analogous to tuberculin, and the final product, "tuberculo-albumen." Deyke and Much state that Filtrate A and its derivatives are frequently lacking in antigenic properties, and this proved to be the case with us. Although "tuberculo-albumen," gave weakly positive Biuret and Tanret's reactions, we are inclined to believe that it consists chiefly of insoluble material—detritus and cellulose. It seemed probable that the several alcohol and ether extractions had together removed all the active antigenic substances from the original mass. The several preparations we made were all strongly anticomplementary.

There remained, however, from the lactic acid digestion five good antigens—Filtrates A.E., A.F., A.G., A.H., and A.I., supposedly representing respectively the four fatty acids and the neutral fats of the tubercle bacilli. Each antigen also unquestionably contained a certain amount of the tubercle bacilli protein. In the several prepara-

tions that we have made of partial antigens by lactic acid digestion, these five antigens have constantly proved of value, while in no instance have we obtained good antigens from either Filtrate A or the tuberculo-albumen. Indeed, we are inclined to believe that in these five antigens we have practically all of the antigenic substances from the tubercle bacillus.<sup>3</sup>

We do not believe these several antigens to be pure partial antigens, either in the chemical or biologic meaning of the word. Certainly all contain traces of protein, and in a measure they overlap each other. Yet each separate antigen is chiefly composed of the various fatty acids or neutral fats (in the case of Filtrate A.I.). This point will be further discussed.

With all sera tested, we have also employed a whole bacillus antigen. For this we have used a preparation somewhat similar to Koch's "Bazillen Emulsion." This was prepared by emulsifying 5 grams of killed and washed tubercle bacilli in 500 cc. of a mixture of equal parts of glycerin and salt solution, to which 2.5 cc. of phenol was added. This mixture was shaken at high speed in a shaking machine for three days, producing a fine bacillary emulsion. This emulsion was titrated as previously described, and found to be a valuable antigen.

### *Complement fixation*

1. *Preparation of sera.* Blood was collected and the serum removed in the usual manner and inactivated at 59 to 60°.<sup>4</sup> To remove any natural amboceptor, 4 cc. of a 2 per cent suspension of goat erythrocytes were added to a 1 cc. of the inactivated serum. This was incubated for one hour at 38°, the cells thrown down by centrifuging, and the dilute serum pipetted off. This 20 per cent serum dilution was used in the test.

2. *Hemolytic system.* Owing to the fact that it is stated that rabbit serum frequently contains natural amboceptor for the partial antigens, we have used a calf anti-goat erythrocyte amboceptor. This was obtained by the repeated intravenous injection of goat erythrocytes

<sup>3</sup> Deyke and Much state that it is necessary frequently to titrate the several antigens, as the titer gradually changes. In our work the antigens used were re-titrated about every ten days.

<sup>4</sup> Owing to the altitude of Fort Bayard, 6000 feet, it was found necessary to heat the sera to 59 to 60° in order to obtain complete inactivation. Likewise all incubation was done at 38°.

into young, tuberculin negative steers. Sensitized cells were used in all tests.

Complement, normal guinea-pig serum, was collected and titrated on the morning of the tests. Complement was always titrated in the presence of one or another of the partial antigens. Complement was always used in the next below the last dilution showing complete hemolysis.

*Technique of the reaction.* In order to quantitate the strength of the reaction, three different quantities of serum, 0.2, 0.5, 0.8, were used with each antigen. One-half cubic centimeter of titrated and properly diluted complement, 0.5 cc. of titrated and diluted antigen, and sufficient salt solution to bring the total volume up to 2.0 cc. were added to each tube. The tubes were then incubated for one hour, and 1.0 cc. of sensitized cells added. The tubes were then returned to the water bath for three hours and the results then read. The usual serum, haemolytic, and antigen controls were run in every instance.

A +++ reading indicated complete, or practically complete hemolysis with all quantities of serum. A ++ result indicated similar fixation in the tubes containing the two larger quantities of serum, while a + sign indicated that such fixation of complement was observed only in the tube containing the maximum amount of serum. In reading the results, 50 per cent inhibition of hemolysis, and under, were read as negative.

#### RESULTS OF COMPLEMENT FIXATION

The sera tested have been grouped in five classes: normal individuals, inactive or arrested cases of tuberculosis, incipient tuberculosis, active cases, and advanced cases. Wassermann reactions were performed upon the blood serum whenever possible.



TABLE 1  
*Normal individuals*

[illegible]

TABLE 1—*Continued*

SERUM NUMBER	BACILLEN EMULSION	FILTRATE A. E.	FILTRATE A. F.	FILTRATE A. G.	FILTRATE A. H.	FILTRATE A. I.	WASSER- MANN REACTION
43	neg.	neg.	+	neg.	neg.	neg.	neg.
44	neg.	neg.	neg.	neg.	neg.	neg.	neg.
45	neg.	neg.	+++	+++	neg.	neg.	+++
46	neg.	neg.	neg.	+++	+	neg.	neg.
47	neg.	neg.	+	+++	neg.	neg.	++
48	neg.	neg.	neg.	neg.	neg.	neg.	neg.
49	neg.	neg.	neg.	neg.	neg.	neg.	neg.
50	neg.	neg.	neg.	+++	++	neg.	+++

TABLE 2

*Inactive tuberculosis*

SERUM NUMBER	BACILLEN EMULSION	FILTRATE A. E.	FILTRATE A. F.	FILTRATE A. G.	FILTRATE A. H.	FILTRATE A. I.	WASSER- MANN REACTION
1	+	neg.	neg.	+	neg.	+++	neg.
2	neg.	neg.	neg.	+	neg.	neg.	neg.
3	neg.	neg.	neg.	neg.	neg.	neg.	neg.
4	neg.	neg.	neg.	neg.	neg.	neg.	neg.
5	neg.	neg.	neg.	+	neg.	neg.	neg.
6	neg.	neg.	neg.	neg.	neg.	neg.	neg.
7	neg.	neg.	neg.	neg.	neg.	+	neg.
8	neg.	neg.	neg.	neg.	neg.	neg.	=
9	neg.	neg.	neg.	neg.	neg.	neg.	neg.
10	neg.	neg.	neg.	+	neg.	neg.	neg.
11	neg.	neg.	neg.	neg.	neg.	neg.	neg.
12	neg.	neg.	neg.	++	+	neg.	neg.
13	neg.	neg.	neg.	neg.	neg.	neg.	neg.
14	neg.	neg.	neg.	neg.	neg.	neg.	neg.
15	neg.	neg.	neg.	+	neg.	neg.	++
16	neg.	neg.	neg.	neg.	neg.	neg.	neg.
17	neg.	neg.	neg.	neg.	neg.	neg.	neg.
18	neg.	neg.	+	neg.	neg.	neg.	neg.
19	neg.	neg.	neg.	neg.	neg.	neg.	neg.
20	+	neg.	neg.	neg.	neg.	neg.	neg.
21	neg.	neg.	neg.	neg.	neg.	neg.	neg.
22	neg.	+	neg.	neg.	neg.	neg.	neg.

TABLE 3  
*Incipient tuberculosis*

SERUM NUM- BER	BACILLEN EMULSION	FILTRATE A. E.	FILTRATE A. F.	FILTRATE A. G.	FILTRATE A. H.	FILTRATE A. I.	WASSERMANN REACTION	REMARKS
1	neg.	+	++	neg.	neg.	+		
2	+	neg.	neg.	++	neg.	+		
3	++	neg.	+	neg.	+++	neg.		
4	neg.	+	neg.	+	+	neg.		
5	neg.	neg.	+	neg.	neg.	neg.	neg.	Case doubtful clinically.
6	neg.	++	neg.	+	neg.	neg.	neg.	
7	neg.	+	neg.	neg.	neg.	neg.	neg.	{ No physical signs. Sputum neg. Hemorrhages. Physical signs. Sputum neg.
8	neg.	+++	neg.	+	neg.	neg.	neg.	
9	+	+	neg.	neg.	neg.	+		
10	neg.	neg.	++	neg.	neg.	+		
11	neg.	neg.	++	neg.	neg.	+		
12	neg.	+	+	neg.	neg.	neg.		
13	neg.	++	+	neg.	neg.	neg.	neg.	
14	neg.	+	++	neg.	neg.	+		
15	+	neg.	neg.	neg.	neg.	+	neg.	{ No physical signs. Neg. sputum.
16	++	+++	+	+	neg.	neg.	neg.	
17	+	neg.	+	+	neg.	+	neg.	
18	neg.	neg.	neg.	+++	+++	neg.	++	
19	neg.	neg.	+++	+++	neg.	neg.	+	
20	neg.	+	neg.	neg.	+	neg.		
21	++	++	+	+++	++	+	neg.	
22	neg.	+	neg.	neg.	neg.	neg.	neg.	
23	+	++	neg.	+++	neg.	+	+	
24	+	++	neg.	++	+	++	+	
25	neg.	++	neg.	++	++	++	++	
26	+	+	+	neg.	neg.	+	+	
27	neg.	+	++	+	++	+		
28	+	++	neg.	neg.	+	+		

TABLE 4  
*Active tuberculosis*

SERUM NUMBER	BACILLEN EMULSION	FILTRATE A. E.	FILTRATE A. F.	FILTRATE A. G.	FILTRATE A. H.	FILTRATE A. I.	WASSERMANN REACTION	REMARKS
1	+++	++	++	+	++	++		
2	+	++	+++	neg.	+	neg.		
3	neg.	+	+	neg.	neg.	neg.		
4	++	+++	+++	+++	+++	+		
5	+++	+++	++	++	+++	+		
6	+	+++	neg.	neg.	neg.	neg.	neg.	
7	+	+	+	neg.	+++	+	neg.	
8	neg.	neg.	neg.	neg.	neg.	+		
9	neg.	++	+	neg.	neg.	neg.	neg.	
10	+	neg.	neg.	++	neg.	+	neg.	
11	+++	++	++	+	++	++	neg.	
12	++	neg.	+	neg.	+++	neg.	No record	
13	+	++	+++	neg.	+	neg.	+	
14	neg.	+	+	neg.	neg.	neg.		Slight activity
15	neg.	+	++	neg.	neg.	+		
16	+++	+++	++	++	+++	+	++	
17	++	++	neg.	neg.	neg.	+		
18	+	+++	neg.	+++	neg.	+	neg.	
19	+	++	+++	+	neg.	+		
20	neg.	neg.	neg.	neg.	neg.	neg.	neg.	
21	+	++	neg.	neg.	neg.	neg.	neg.	
22	++	+++	+++	++	neg.	neg.	neg.	
23	neg.	neg.	neg.	neg.	neg.	neg.	neg.	
24	neg.	neg.	+	neg.	neg.	neg.	neg.	
25	+	++	++	neg.	neg.	neg.	neg.	
26	++	++	+	neg.	++	neg.	neg.	
27	++	++	++	+	neg.	neg.	neg.	
28	neg.	neg.	++	++	++	neg.	+++	
29	++	+++	++	++	+	neg.	neg.	
30	+	neg.	+	neg.	neg.	neg.	neg.	
31	neg.	neg.	neg.	+	neg.	neg.	neg.	
32	+	+	++	neg.	+	neg.	neg.	
33	+	+	++	neg.	neg.	neg.	neg.	
34	+++	+++	+++	+++	+++	+++	+	
35	neg.	neg.	+	+	neg.	neg.		
36	neg.	++	neg.	+++	++	neg.		
37	neg.	neg.	neg.	+++	+++	++	neg.	
38	+	neg.	neg.	++	neg.	neg.		
39	+	++	+	neg.	+	+		

TABLE 5  
*Advanced tuberculosis*

SERUM NUMBER	BACILLEN EMULSION	FILTRATE A. E.	FILTRATE A. F.	FILTRATE A. G.	FILTRATE A. H.	FILTRATE A. I.	WASSER- MANN REACTION	REMARKS
1	++++	+	++	+++	+++	+++		
2	++++	++++	+++	+++	+++	+++		
3	++	++++	+++	++	++	neg.		
4	+	++++	++	neg.	neg.	++		
5	++++	++++	++	+++	+++	+++		
6	++++	++++	+++	+++	++	++		
7	neg.	++++	++	+++	neg.	+	±	
8	neg.	neg.	neg.	neg.	neg.	neg.		
9	neg.	neg.	neg.	neg.	neg.	neg.	neg.	
10	neg.	+++	neg.	neg.	neg.	+	neg.	
11	+	neg.	++	neg.	neg.	+	neg.	
12	++++	++	+++	+++	+++	+		
13	++++	+++	+++	++	+	+		Dead
14	++++	+	++	+++	+++	+++		
15	++++	+++	+++	+++	+++	+++	neg.	
16	+	neg.	neg.	+	neg.	+++		Dead
17	++	++++	+++	++	+++	+		
18	++	++++	+++	neg.	neg.	++		Dead
19	+	++++	++	neg.	+++	+++		
20	++++	+++	++	+++	++	++		
21	+++	+++	+++	+++	++	++		
22	neg.	neg.	neg.	neg.	neg.	neg.		
23	+++	+++	+++	++	++	++	neg.	
24	+	+++	neg.	neg.	neg.	++	++	
25	+	+++	neg.	neg.	+++	neg.	neg.	
26	++	+++	+++	+++	+++	+	neg.	
27	+	++	+	+++	+	neg.	neg.	
28	+	+++	++	+	++	neg.	neg.	
29	+	+	++	neg.	neg.	neg.	+	
30	+	++	+	neg.	++	neg.	neg.	
31	++	++	++	+++	++	+	±	
32	neg.	neg.	neg.	+++	+++	+	neg.	
33	+	+++	+++	++	+++	+	neg.	
34	neg.	neg.	+	+++	+++	++	neg.	
35	+	+++	+++	+++	+++	++		
36	neg.	neg.	+++	++	++	++	neg.	
37	++	++	+++	+++	+++	++	neg.	

## DISCUSSION

*Analysis and clinical significance of results*

The results of this work may be summarized as follows: The sera of 176 persons have been examined with the complement binding reaction against each of the five partial antigens, and against a bacillen emulsion. Of these 176 sera, 50 were from persons clinically free from tuberculosis, and of these 50, 9 reacted positively to one or more of the partial antigens. Twenty-two sera were from persons formerly clinically tuberculous, but who for one year had been free from clinical evidence of the disease. In this group 9 cases gave a positive reaction with one or another of the antigens, but only 2 sera, or 9 per cent gave a reaction of any strength. Twenty-eight sera were from cases classed clinically as incipient tuberculosis. These cases showed only a slight involvement, but from the clinical evidence they were definitely diagnosed as active tuberculosis and were named as Class I, Involvement I.<sup>5</sup> Of these cases only 3 gave what could be considered negative reactions, while even these three gave a weakly positive reaction with at least one of the several antigens.

Thirty-nine sera were from cases of clinically active tuberculosis, carried as Class II, Involvement II. Thirty-four, or 87 per cent, of these gave strongly positive results, 4 gave weakly positive results with one or more of the antigens, and one case, was negative with all the antigens. The remaining 37 sera were from patients with advanced tuberculosis, carried as Class III, Involvement III. Of these 34 gave intensely strong reactions, while the remaining 3 gave negative reactions.

Reviewing these results of the complement-binding reaction more closely: in 50 normal cases 8 had a positive reaction. Of these 8 cases, 3 are civilians who have not been in direct contact with the patients of this hospital; the remainder are soldiers, of whom 2 have had little to do with the sick, and 2 have been on duty as nurses and orderlies. Of the remaining 42 cases,

<sup>5</sup> Patients are classified according to "Class," referring to prognosis, and "Involvement," referring to the degree of tuberculous involvement present.

in 28 cases the subjects have been on duty which has brought them into more or less intimate contact with tuberculosis.

Of the 22 cases of tuberculosis classed as inactive, 2 had a positive reaction. In one there was a recent history of positive sputum; the other is an old case with negative sputum but with an extensive, though, as far as can be determined by physical signs, at present an inactive lesion.

In 28 cases classed as incipient or, more correctly, cases with apparently small lesions, 3 cases gave a negative reaction. Of these, in one the diagnosis of tuberculosis was made on the presence of subcrepitant râles and repeated hemoptyses, for the sputum has always been negative. In the second the salient symptoms were severe pains in the chest coming on after exposure and repeated hemoptyses, the hemorrhages continuing until artificial pneumothorax was performed for their arrest. In both of these cases the diagnosis is doubtful. The third case is one of old pulmonary tuberculosis which might be called inactive.

Of 39 cases classed as active, 5 gave a negative reaction. Of these, 4 are very old cases of tuberculosis with marked contraction of the upper lobes as shown by absence, or great narrowing of Krönig's isthmus, or with extreme sharpness of the X-ray shadows. The signs relied upon as evidencing activity of the tuberculous process may not warrant this interpretation. The fifth case is one of interest. The patient acquired pleurisy with effusion in 1912, at a time when there was an epidemic of streptococcic bronchitis at his post, Vancouver Barracks, and has been under treatment at this hospital intermittently since that time. Much doubt has been felt as to the diagnosis in this case. It was decided at one time that the pleurisy was not tuberculous. Finally the sputum became positive for tubercle bacilli, but the good health of the patient in connection with negative complement-binding reaction seems to show that his extensive pulmonary lesion is not ascribable entirely to tuberculosis, but that the tuberculosis is a comparatively unimportant feature in the case.

Three out of 37 advanced cases of tuberculosis gave a negative

complement-binding reaction. Two of these are very old cases in fairly good condition; the third is a far advanced case in which the negative reaction is probably a bad omen.

While the result of the complement-binding reaction is not what might have been expected in these exceptional cases, on the whole the findings agree very well with the clinical facts. The physical signs that are supposed to denote activity of a tuberculous process are not reliable in very chronic cases. It is quite possible that further study of the complement-binding reaction may show that it is capable of supplementing the physical diagnosis in a very useful way if it can help to decide the significance of a "moist" lung in an afebrile subject.

The complement-binding reaction with the partial antigens in the main, therefore, conforms very closely with the clinical findings, and seems, from the evidence we have, to be of some value as a diagnostic means, especially in cases of early or incipient tuberculosis.

No one antigen seems to us to be of especial value, nor does the intensity of the reaction to one antigen seem to bear any relationship to the degree of activity. On the other hand, in reviewing the results given by the various groups of patients, the degree of intensity of positive reactions to the antigens as a group seems to us to run remarkably parallel to the degree of tuberculous activity present in the several groups of patients. In the incipient cases the reactions were as a rule weaker, and negative reactions with one or more of the partial antigens were more frequently seen than in cases in which the tuberculous process was more advanced. Similarly the cases classed as active tuberculosis gave, in general, stronger reactions and fewer negative reactions than the incipient cases. The cases classed as advanced tuberculosis in turn gave almost uniformly triple-plus reactions, and negative reactions with any single antigen were somewhat the exception. While we hardly believe that in any given case we are justified, with the evidence at hand, in deciding the degree of activity from the intensity of the reactions to the partial antigens, nevertheless the degree of tuberculous activity in the classes of patients examined does



seem to run in general surprisingly close to the intensity of the reaction to the partial antigens as a group. That the great majority of cases of clinically active tuberculosis should give a positive complement-binding reaction to tuberculous antigens is what would be expected. The interest in the application of the reaction to this class of cases lies chiefly in the study of the presence or absence of antibodies to the individual partial antigens in the given case. On this phase of the subject our observations are necessarily defective on account of the failure to obtain all the antigens in a pure state. With reference to the occurrence of the reaction in the clinically healthy, disappointment has been felt that the reaction is not limited to cases of demonstrably active tuberculosis and many investigators have labored to secure a tuberculous antigen which shall not be too sensitive. Leschke (17) says:

The diagnostic hopes placed upon the demonstration of complement-antibodies have not been fulfilled, could not be fulfilled because the antibodies point only to a previous contact with tuberculosis, not to tuberculous disease. The frequent occurrence of specific complement-binding antibodies in those clinically free from tuberculosis in agreement with the presence of tuberculin sensitiveness in healthy adults, serves to support the view that a high percentage of all adults have come in contact at some time with the virus of tuberculosis and have thereby attained a more or less perfect immunity to tuberculosis.

But if we accept, as we apparently must, the view of Much that the presence of humoral antibodies denotes a condition of active resistance on the part of the organism to tuberculous virus, we must believe that every positive complement-binding reaction means the presence of an active tuberculous focus, however healthy the individual may appear to be. The fact is that in a race almost universally brought into contact with tuberculosis, in which immunity is obtained at the price of a reaction more or less intense against tubercle bacilli, there is not the sharp distinction between the clinically well and the clinically tuberculous that many attempt to draw in practice. We are just beginning to realize the existence of one stage in the development of tuber-

culosis in the partially immune subject which is revealed chiefly by the X-ray—the stage of lymphatic extensions from the hilus, deep peribronchial tuberculosis. It is altogether probable that the positive reaction in the apparently healthy is an indication of the activity of a deep seated focus, although the subject, if advancing in the direction of a more complete immunization, may never furnish the ordinary clinical evidences of active tuberculosis. If this view shall be found to be correct, the positive complement-binding reaction may prove of inestimable value in indicating the necessity for especial care of the individual, however healthy he may seem, in order that the process may progress in the proper direction, and may lead to a more complete immunization rather than to a clinically apparent pulmonary tuberculosis.

While there can be no doubt that the complement-binding reaction indicates an active tuberculous focus in many cases, there is another possibility that should be considered. Much has several times referred to the case of his assistant Altstaedt, who being placed for the first time in charge of a tuberculous ward was found to have no tuberculous antibodies in his blood when he first began his work; at a later time his blood gave a positive reaction to all the partial antigens, without, however, the development of clinically apparent tuberculosis. The inference drawn from these facts seems to be that here exposure to tuberculous virus from without the organism led to the formation of humoral antibodies in an immune subject. This view raises a question of immense importance in the epidemiology of tuberculosis: the question as to how immunity is maintained in exposure to infectious material from without. Does the immune organism find it necessary to call forth its reserves, the humoral antibodies, to prevent a super-infection? If this is the case the tuberculosis nurse, the laboratory worker, the phthisiologist, the laryngologist, who spend their professional lives in more or less constant contact with tuberculous virus, should show a positive complement-binding reaction with tuberculous antigens. Whether they do or not is easily to be determined by investigation. We venture to predict that it will be found that

as a class such workers will furnish negative results. Just as tuberculin sensitiveness is found more frequently and more markedly in the young and indicates a stage of resistance in which, so to speak, a struggle to maintain one's ground is apparent, while in seasoned immunity a tuberculin reaction is only excited with difficulty after repeated trials and larger doses, so it is probable that the presence of humoral antibodies is an evidence that immunity against the original infection is not yet so perfect that it can be maintained without a struggle.

#### COMPOSITION OF ANTIGENS

As we have before stated, we do not believe the antigens we have used in this work to be strictly "partial" antigens, either in the biologic or chemical sense of the word. Rather do we believe them to contain, in traces at least, several of the various chemical constituents that make up the tubercle bacillus. They are partial antigens only in the broadest sense of the word, rough groupings of the various fatty acids, possibly overlapping each other, and all containing a trace of protein. Filtrates A.E., A.F., A.G., and A.H. are undoubtedly composed chiefly of the fatty acids soluble in absolute alcohol at 0°, 37°, 56°, and 78° respectively. Microscopically they seem to be entirely fatty acid crystals, yet we know that they contain traces of protein. To assume that the fatty acids contained in the various antigens are completely different in the different antigens, is to assume that the extractions at the various temperatures were each one complete, a factor of which we cannot be sure. Moreover, it is a well known fact in physiological chemistry that a simple alcoholic extraction of a mass of protein and lipoids, will not yield a pure lipid, but that a certain amount of protein is also extracted with the lipoids.

This brings us naturally to the question as to whether or not the various fatty acids and lipoids contained in the antigens are in any way responsible for their antigenic properties; that is, whether the antigenic properties of the various antigens are not due entirely to their protein content.

The well known experiments of Bang and Forsman (18) indicate the lipoids may possess specific antigenic properties. Dautwitz and Landsteiner (19), while confirming Landsteiner's observations, suggest that the apparent antigenic properties of lipoids may be due to other products present in a colloidal suspension. Although Dudgeon (20), Meek (22) and Weir (21) obtained complement fixation with tuberculous sera, with an alcoholic extract of tubercle bacilli as antigen, it is probable their antigen contained protein. Meyer (23) obtained no fixation of complement with tuberculous sera with antigens representing the fatty acid and neutral fat fractions of the tubercle bacillus. On the other hand, Bronfenbrenner (24) found that the lipin fraction of Besredka's antigen caused constant deviation of complement in lipotropic sera. Lucke (25) found that a purified tubercular wax caused deviation of complement, but this deviation was not specific for tuberculous sera.

Landsteiner (26) suggests that lipoids go into solution in a lipid-protein combination, and that the lipoids by their mere presence may alter the solubility of the protein. Jobling (27) also suggests that "lipoids in certain combinations may act as antigens, while pure lipoids have not that property." This is the view we are forced to take. The reactions of the same serum often differed with the various partial antigens. It is improbable that the protein elements of the various antigens differed, while we know the lipid and fatty acid content was, in the main, different. It seems probable, therefore, that the protein is present in a protein-lipoid combination, and that this combination has specific antigenic properties, differing as the lipid element, or possibly the protein element, in the combination differs.

The criticism has been advanced that if these reactions are due to the fatty acids or neutral fats, can the reaction be considered in any way specific? We cannot answer this. If our views before mentioned are true—that the reactions are due to a lipid-protein combination, and that they differ as the lipid content or possibly the protein content of the combination varies—it is of little moment whether or not the lipid content of the tubercle bacillus is specific.

RELATIONSHIP OF THE TUBERCULOSIS COMPLEMENT-BINDING  
REACTION TO THE WASSERMANN REACTION TEST<sup>6</sup>

Owing to our remoteness from large general hospitals we have found it impossible to obtain any appreciable number of syphilitic sera, and for that reason no attempt has been made to determine the percentage of syphilitic sera that react positively to the partial antigens. A Wassermann test was performed in 126 of the 176 cases reported and 27 of the 126 gave a positive reaction. Twelve of these positive syphilitic sera were from individuals classed as normal as regards to tuberculosis, and of these 12, 5 reacted positively to the partial antigens. Two positive syphilitic sera were from patients classed as "inactive tuberculosis," and both of these sera gave negative results with the partial antigens. The remaining thirteen syphilitic sera were from clinically tuberculous patients, and all of these reacted positively to the partial antigens.

The relation of syphilis to the complement-binding reaction with tuberculous antigens is a subject which urgently demands further elucidation. The observations in our series are too few in number to permit the drawing of any definite conclusions. The almost universal prevalence of tuberculous infection (as distinguished from tuberculous disease) and the absolute insensitiveness of the truly non-tuberculous to tuberculin would, however, seem to show that the reaction of syphilitic cases in apparently a large percentage to tuberculous antigens is to be explained by supposing that the victims of another infection do not resist the tuberculous infection as well as the average, rather than by the assumption of a lack of specificity in the complement-binding reaction to tuberculous antigens.

<sup>6</sup> Snow and Cooper (28), in this laboratory, have previously reported their findings in regard to the relationship of the tuberculosis complement fixation test to the Wassermann reaction. The authors found frequent non-specific fixation, especially with a cholesterin reinforced antigen. As a result of this work, a less sensitive antigen, a simple alcoholic extract of human heart, has since been used in this laboratory in the routine Wassermann reaction.

## SUMMARY

Partial antigens have been prepared from the tubercle bacillus, by disintegrating the bacilli in 1 per cent lactic acid, filtering, and extracting with alcohol, at varying temperatures, and with ether. The resulting antigens appear to be composed chiefly of the various fatty acids, and neutral fat of the tubercle bacillus together with traces of protein.

One hundred and seventy-six normal and tuberculous sera were titrated against these partial antigens, and against whole bacillus emulsion antigen. Seventy-two of the sera obtained from clinically normal cases, and 91 per cent of the sera from cases classed clinically as inactive tuberculosis reacted negatively to these antigens. Ninety per cent of the sera from cases classed clinically as incipient tuberculosis, 87 per cent of the sera from cases classed as active tuberculosis, and 92 per cent of the sera from advanced tuberculous cases reacted positively to the partial antigens.

Sufficient syphilitic sera could not be obtained to make any determination of the proportion of syphilitic sera which would react positively to these antigens but judging from 27 positive Wassermanns obtained at the same time the sera were examined, there appears to be little relationship between the Wassermann reaction and the reaction to the partial antigens.

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## COMPLEMENT FIXATION IN ACUTE ANTERIOR POLIOMYELITIS<sup>1</sup>

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During the epidemic of acute anterior poliomyelitis of the past summer we undertook as part of a series of studies in this disease with the cooperation of Dr. Charles K. Mills, the study of complement fixation with the cerebrospinal fluids and blood sera of persons in different stages of the disease and various tissues and bacterial antigens, to determine first, whether specific antigen could be detected by this means in the tissues of the central nervous system and other organs and secondly, whether antibodies for the various and easily cultivated bacteria found in the cerebrospinal fluid and various organs could be demonstrated by this method in the cerebrospinal fluid and blood serum.

The numerous transmission experiments of Flexner and his associates and others have proven that the virus of this disease is present in the brain, cord, various lymphatic glands and spleen and that antibodies are developed inasmuch as one attack of the disease appears to confer an immunity and the sera of convalescents possesses the power of neutralizing or destroying the virus in vitro and conferring passive immunity. Nothing further is definitely known of the immunity in the disease; the complement fixation experiments of Wollstein (1) using extracts of medulla, spinal cord, sciatic nerve, tibial muscle and cerebrospinal fluid as antigens, yielded uniformly negative results in a small series of cases. Lebrede and Recio (2) claim to have obtained a fixation reaction in one of three cases tested, using

<sup>1</sup> Part of this paper was read in the symposium upon acute anterior poliomyelitis before the Philadelphia County Medical Society, November 6, 1916.

extracts of various organs as antigens, while Gay and Lucas (3) in a series of complement-fixation tests found no evidence of antibodies or antigen in the spinal fluid and blood serum of poliomyelitic monkeys or human beings.

We have prepared salt solution and alcoholic extracts of many different tissues secured at autopsy from typical cases of acute anterior poliomyelitis, using large amounts of tissue in order to concentrate the virus in our antigen extracts; also salt solution suspensions of the streptococci, diplococci, diphtheroids and Gram-negative bacilli cultivated during life from the cerebrospinal fluid of persons with acute poliomyelitis and from various tissues secured at autopsy (4), and have used them after preliminary titrations, in complement fixation tests with the cerebrospinal fluids and sera of persons during and after an attack of acute anterior poliomyelitis and of persons that have never had the disease.

#### TECHNIC AND METHOD OF STUDY

*Preparation of salt solution extracts of tissues.* The tissues were secured at autopsy and used immediately; 10 grams were thoroughly ground in a mortar with sterile sand and mixed with 100 cc. of sterile physiological salt solution containing 0.5 per cent phenol. The emulsion was shaken mechanically for four hours; incubated at 37°C. for four days; centrifuged, filtered through sterile paper and the filtrate used as antigen after preliminary titration.

*Preparation of alcoholic extracts of tissues.* These extracts were prepared with absolute alcohol in the same manner except that incubation was continued for about ten days in order to facilitate extraction. These extracts were diluted with physiological salt solution before titration and use in the complement fixation tests.

*Preparation of bacterial antigens.* Polyvalent antigens were prepared of the streptococci, diplococci, diphtheroids and Gram-negative bacilli recovered from the cerebrospinal fluids and tissues of numerous cases, by cultivating pure cultures in large volumes of ascites-kidney broth, centrifuging the supernatant fluid until a heavy sediment of bacteria was obtained and washing the bacteria once with physiological salt solution by the same means, followed by re-suspension in physiological salt solution, heating at 60°C. for one hour and preserving with 0.5 per cent phenol.

*Antigen titration.* All antigens used in the different experiments were sufficiently diluted with physiological salt solution, titrated just before the complement fixation tests were conducted, and employed in amounts equal to one-half or one-third the anticomplementary units; these amounts were large but we purposely used them in order to increase the delicacy of the reactions, as it is well known that complement fixation in all diseases other than syphilis is apt to be of slight degree.

*Complement fixation tests.* The antisheep hemolytic system was employed; 1 cc. of a 1:20 dilution of complement serum of four or more pigs and two units of hemolysin was employed throughout in the antigen titrations and complement fixation tests. Primary incubation was of one hour duration in the thermostat; secondary incubation was of the same duration or until the controls showed complete hemolysis. *The readings were made immediately after the second period of incubation*, which was of considerable importance inasmuch as weakly positive reactions tended to show complete hemolysis after standing over night due to the presence of an excess of hemolysin.

The cerebrospinal fluids were used unheated and in a constant and rather large dose, namely, 1 cc.; the sera were heated at 56°C. for thirty minutes and used in constant dose of 0.2 cc.

In every experiment each antigen, cerebrospinal fluid and serum were controlled and unless hemolysis was complete the results were excluded.

#### *Antigens employed*

Three different salt solution extracts were prepared of poliomyelitic spinal cord, pons and medulla, cerebellum, cerebrum, spleen and liver; one extract each of poliomyelitic tonsils, mesenteric glands, suprarenal glands, thymus gland and pancreas.

Control antigens were prepared of rabbit liver and rabbit spleen and non-poliomyelitic human liver and spleen.

Alcoholic extracts were prepared and used of each of the following: poliomyelitic cord, pons, cerebellum, cerebrum, liver and spleen; also one each of rabbit liver and spleen and non-poliomyelitic human liver and spleen.

The following bacterial antigens were employed:

(a) A polyvalent suspension of four cultures of diplococci cultivated from two cerebrospinal fluids, one cord and one cerebrum.

(b) A polyvalent suspension of fifteen cultures of streptococci from poliomyelitic cords, pons, cerebrum, thymus gland and spleen.

(c) A polyvalent suspension of three cultures of diphtheroids from cerebrospinal fluids.

(d) A polyvalent suspension of six cultures of Gram-negative bacilli from cerebrospinal fluids and spinal cords.

Wassermann reactions were also conducted with each cerebrospinal fluid and serum with an antigen of acetone insoluble lipoids of human heart in dose of 0.2 cc. of 1:20 dilution. This dose was double the antigenic unit and twelve times less than the anticomplementary dose.

The various tissue extracts varied considerably in antigenic sensitiveness and those possessing some antigenic value usually deteriorated within ten days to two weeks after standing in the refrigerator.

#### RESULTS OF COMPLEMENT FIXATION TESTS WITH CEREBROSPINAL FLUIDS

The fluids of 130 patients were examined; the patients varied in age from six months to twenty-seven years and the fluids were collected aseptically at intervals varying from one to thirty-eight days *after the onset of paralysis*. In a number of instances the fluid of the same patient was tested more than once.

The results are summarized in table 1.

1. Positive reactions were observed with 6 to 16 per cent of fluids with salt solution antigens of poliomyelitic cord and brain tissue.

2. The majority of these reactions occurred after the fifth day following the onset of paralysis, but there was no constant relation between the duration or severity of the disease and the complement fixation reactions.

3. Negative reactions resulted with the extracts of tonsils, thymus gland, suprarenal glands, mesenteric glands and pancreas due, probably, to high dilution of the virus and to the fact that but one set of tissues (tonsils from three cases) were used in the preparation of the extracts.

4. In all instances the degree of complement fixation was slight and not over a ++ or 50 per cent inhibition of hemolysis.

5. The alcoholic extracts of these tissues yielded negative reactions.

6. A surprisingly high percentage of positive reactions was observed with the salt solution extracts of poliomyelitic spleen and liver. We have no explanation to offer for these reactions; all extracts were carefully titrated preliminary to the complement fixation tests and in all instances the controls showed complete hemolysis.

TABLE 1

*Summary of complement fixation reactions in acute anterior poliomyelitis with cerebrospinal fluids and antigens of poliomyelitic tissues*

ANTIGEN OF	KIND OF EXTRACT	TOTAL NUMBER OF FLUIDS TESTED	RESULTS		PERCENTAGE OF POSITIVE REACTIONS
			Positive reactions	Negative reactions	
Cord.....	NaCl	123	14	109	10
Pons and medulla.....	NaCl	82	8	74	9
Cerebellum.....	NaCl	77	5	72	6
Cerebrum.....	NaCl	87	14	73	16
Tonsils.....	NaCl	80	0	80	0
Spleen.....	NaCl	155	26	129	17
Liver.....	NaCl	147	22	125	15
Mesenteric glands.....	Alcoholic	12	0	12	0
Suprarenal glands.....	Alcoholic	12	0	12	0
Thymus glands.....	Alcoholic	12	0	12	0
Pancreas.....	Alcoholic	12	0	12	0
Cord.....	Alcoholic	24	0	24	0
Pons and medulla.....	Alcoholic	24	0	24	0
Cerebellum.....	Alcoholic	24	0	24	0
Cerebrum.....	Alcoholic	24	0	24	0
Spleen.....	Alcoholic	32	0	30	0
Liver.....	Alcoholic	32	0	30	0

7. Control tests with poliomyelitic fluids and extracts of human and rabbit non-poliomyelitic liver and spleen, yielded much lower percentages of positive reactions although slightly positive reactions occurred with many of the fluids which gave stronger reactions with corresponding extracts of poliomyelitic tissues (table 2).

8. All of the fluids yielding these slightly positive reactions with various tissue antigens, yielded negative Wassermann reactions.

TABLE 2

*Summary of complement fixation reactions in acute anterior poliomyelitis with cerebrospinal fluids and antigens of non-poliomyelitic tissues*

ANTIGEN OF	KIND OF EXTRACT	TOTAL NUMBER OF FLUIDS TESTED	RESULTS		PERCENTAGE OF POSITIVE REACTIONS
			Positive reactions	Negative reactions	
Human spleen.....	NaCl	57	3	54	5
Human liver.....	NaCl	22	0	22	0
Rabbit spleen.....	NaCl	42	3	39	7
Rabbit liver.....	NaCl	46	0	46	0
Rabbit spleen.....	Alcoholic	24	0	24	0
Rabbit liver.....	Alcoholic	46	0	46	0

9. The cerebrospinal fluids of normal persons or those suffering with diseases other than syphilis yielded negative reactions with all antigens (table 3). The fluids of three cases of meningococcus meningitis yielded a few irregular and weakly positive reactions; while the controls were completely hemolysed, yet the fluids were cloudy with pus cells and cocci, and we are inclined to regard the reactions as non-specific and the summation of the anticomplementary action of fluid and antigens.

10. The fluids of three paretics yielding strongly positive Wassermann reactions with an antigen of acetone insoluble lipoids, yielded strongly positive reactions with all of the antigens of poliomyelitic and non-poliomyelitic tissues (table 3).

#### RESULTS OF COMPLEMENT FIXATION TESTS WITH SERA

The results of these tests summarized in table 4 were practically negative.

With salt solution extracts of poliomyelitic cord and brain, weakly positive reactions were observed with 2 to 4 per cent of sera.

With alcoholic extracts all of the reactions were negative.

Extracts of poliomyelitic liver and spleen did not yield positive reactions with these sera as were observed with many of the cerebrospinal fluids.

TABLE 3  
Summary of complement fixation reactions with the cerebrospinal fluids of non-poliomyelitic persons and various antigens of poliomyelitic and non-poliomyelitic tissues

HISTORY	RESULTS WITH VARIOUS ANTIGENS								
	Aceton insoluble lipids	Cord (NaCl)	Cord (alcoholic)	Cerebrum (NaCl)	Cerebrum (alcoholic)	Spleen (NaCl)	Liver (NaCl)	Normal spleen (NaCl)	Normal liver (NaCl)
Normal.....	-*	-	-	-	-	-	-	-	-
Normal.....	-	-	-	-	-	-	-	-	-
Normal.....	-	-	-	-	-	-	-	-	-
Paresis.....	++	++	++	++	++	++	++	++	++
Paresis.....	++	++	++	++	++	++	++	++	++
Paresis.....	++	++	++	++	++	++	++	++	++
Whooping cough†.....	++	++	++	++	++	++	++	++	++
Whooping cough.....	-	-	-	-	-	-	-	-	-
Whooping cough.....	-	-	-	-	-	-	-	-	-
Measles.....	-	-	-	-	-	-	-	-	-
Measles.....	-	-	-	-	-	-	-	-	-
Measles.....	-	-	-	-	-	-	-	-	-
Meningitis.....	-	-	-	-	-	-	-	-	-
Meningitis=	-	++	-	+	-	+	+	+	-
Meningitis=	-	+	-	-	-	+	-	+	+
Meningitis=	-	-	-	-	-	+	+	-	-

\* - = negative.

+ = very weakly positive.

++ = weakly positive.

+++ = moderately positive.

++++ = strongly positive.

† Child suffering with the condition known clinically as meningismus or serous meningitis.

= Epidemic cerebrospinal meningitis (meningococcic).

TABLE 4

*Summary of complement fixation reactions in acute anterior poliomyelitis with sera and antigens of various poliomyelitic, non-poliomyelitic and normal tissues*

ANTIGEN OF	KIND OF EXTRACT	TOTAL EXAMINED	RESULTS		PERCENTAGE OF POSITIVE REACTIONS
			Positive reactions	Negative reactions	
Cord.....	NaCl	57	2	55	3
Pons and medulla.....	NaCl	38	1	37	2
Cerebellum.....	NaCl	33	1	32	3
Cerebrum.....	NaCl	41	2	37	4
Tonsils.....	NaCl	36	0	36	0
Spleen.....	NaCl	58	0	58	0
Normal human spleen.....	NaCl	10	0	10	0
Liver.....	NaCl	52	0	52	0
Normal human liver.....	NaCl	10	0	10	0
Rabbit liver.....	NaCl	30	0	30	0
Cord.....	Alcoholic	10	0	10	0
Pons and medulla.....	Alcoholic	10	0	10	0
Cerebellum.....	Alcoholic	10	0	10	0
Cerebrum.....	Alcoholic	10	0	10	0
Spleen.....	Alcoholic	10	0	10	0
Liver.....	Alcoholic	10	0	10	0

#### RESULTS OF COMPLEMENT FIXATION TESTS WITH CEREBROSPINAL FLUIDS AND SERA AND VARIOUS BACTERIAL ANTIGENS

Of special interest in this connection are the results of these tests with polyvalent salt solution suspensions of diplococci, streptococci, diphtheroids and Gram-negative bacilli isolated from the cerebrospinal fluids of poliomyelitic persons during life and from the cord, brain and various internal organs after death. Jaeger (5), Geirsvold (6), Bülow-Hansen and Harbitz (7), Harbitz and Scheel (8), Pasteur, Foulerton and Maccormac (9), Wollstein (1) and Flexner and Lewis (10) have recovered various cocci and bacilli from the cerebrospinal fluid, or fluid, cord and brain of cases of acute anterior poliomyelitis and while several of these investigators produced lesions and symptoms in rabbits with the cocci regarded as experimental anterior poliomyelitis, yet the consensus of opinion until very recently was to the effect that these bacteria were not to be considered as the etiological



agent of acute anterior poliomyelitis. The recent investigations of Mathers (11), Rosenow, Wheeler and Towne (12) and of Nuzum and Herzog (13) have re-awakened interest in these micrococci inasmuch as they reported the successful inoculation of monkeys, rabbits, guinea-pigs and other animals by the injection of cultures intracerebrally, intravenously and intraperitoneally. Kolmer, Brown and Freese (4) recovered various bacteria and especially a diplococcus and streptococcus from the cerebrospinal fluid, cord, brain and various internal organs of cases of acute anterior poliomyelitis; while the injection of the cocci into rabbits and monkeys produced a low grade meningitis, there were no clinical or histological evidences of anterior poliomyelitis.

There is but little doubt in our opinion that various bacteria and particularly a diplococcus and streptococcus are present not only in the cerebrospinal fluid, cord, pons, cerebellum and cerebrum, but also in the various internal organs as the thymus gland, liver, spleen, suprarenal glands and enlarged mesenteric glands, of cases of acute anterior poliomyelitis. Many of these cultures possess some virulence for the lower animals and therefore it is not improbable that they may produce antibodies during life analogous to the antibodies produced by streptococci during scarlet fever.

The antigens employed in this study were prepared of pure cultures of various micro-organisms recovered from poliomyelitic patients. As it is well known that complement fixation in bacterial diseases is usually weak, we have employed our antigens in doses corresponding to one-half their anticomplementary units. With these relatively large doses, we have observed a small percentage of weakly positive reactions with cerebrospinal fluids and the antigens of diplococci and streptococci, while all tests with sera were negative with all antigens (table 5).

TABLE 5

*Summary of complement fixation reactions with poliomyelitic cerebrospinal fluids and sera and various bacterial antigens*

ANTIGENS	CEREBROSPINAL FLUIDS			BLOOD SERA		
	Number positive	Number negative	Per cent positive	Number positive	Number negative	Per cent positive
Diplococci.....	2	33	6	0	12	0
Streptococci.....	3	32	9	0	12	0
Diphtheroids.....	0	28	0	0	12	0
Gram negative bacilli.....	0	28	0	0	12	0

#### THE WASSERMANN REACTION IN ACUTE ANTERIOR POLIOMYELITIS

The results of Wassermann reactions conducted with an extract of acetone insoluble lipoids and the cerebrospinal fluids and sera of patients in various stages of anterior poliomyelitis, are summarized in table 6.

TABLE 6

*Summary of Wassermann reactions in acute anterior poliomyelitis with cerebrospinal fluids and sera and an antigen of acetone insoluble lipoids*

SUBSTANCE	TOTAL TESTED	RESULTS		PERCENTAGE OF POSITIVE REACTIONS
		Positive reactions	Negative reactions	
Cerebrospinal fluid.....	130	2	128	1.5
Sera.....	48	0	48	0

As shown in this table but two positive reactions were observed with 130 fluids; none of the sera of the 48 patients tested yielded a positive reaction. It is highly probable that the two children yielding positive reactions were cases of congenital syphilis; unfortunately serum tests were not conducted with these patients. It would appear at least, that in acute anterior poliomyelitis itself the Wassermann reaction is uniformly negative.

#### DISCUSSION

The results of these studies indicate that in a complement fixation test rendered very delicate by employing large doses of antigen and cerebrospinal fluid, a small percentage of apparently

true positive reactions occurs with salt solution extracts of the spinal cord, pons and medulla, cerebellum, cerebrum, liver, spleen or other tissues of fatal cases of acute anterior poliomyelitis. Alcohol did not serve to extract antigenic principles from these tissues and this is in agreement with similar studies in other infections.

Of unusual interest in this work were the number of positive reactions with the extracts of liver and spleen and the greater degree of inhibition of hemolysis as compared with the other extracts. A marked variation in antigenic sensitiveness was noted with the extracts of all tissues, but one of those of spleen and of liver yielded a series of unusually strong reactions with the cerebrospinal fluids. In every experiment each extract was carefully titrated beforehand and controlled in the complement fixation tests, as were also all cerebrospinal fluids; therefore we cannot ascribe the results to non-specific fixation of complement.

These results also indicate that the antibody or reactionary substance concerned in the complement fixation reaction is present or, at least more easily detected, in cerebrospinal fluid than in serum; this, however, may be due to quantitative factors. Furthermore it appears that the micrococci so readily and easily cultivated from the cerebrospinal fluid and tissues of acute anterior poliomyelitis may possess sufficient virulence to produce antibodies, although this does not necessarily indicate that they are to be regarded as the cause of the disease.

#### SUMMARY

1. Complement fixation tests conducted with the cerebrospinal fluids of 130 persons suffering with acute anterior poliomyelitis and salt solution extracts of various tissues yielded from 6 to 16 per cent weakly positive reactions with extracts of spinal cord, pons and medulla, cerebellum, cerebrum and 15 to 17 per cent positive reactions with extracts of poliomyelitic liver and spleen. Control antigens of non-poliomyelitic human and rabbit liver yielded negative reactions, while extracts of spleen yielded from 5 to 7 per cent positive reactions.

2. With alcoholic extracts of these tissues the tests were uniformly negative.

3. The cerebrospinal fluids of normal persons and children suffering with whooping cough and measles yielded negative reactions with all extracts. The cerebrospinal fluids of several paretics yielded strongly positive reactions with all salt solution and alcoholic extracts.

4. Complement fixation tests with the sera of 58 persons in the various stages of acute anterior poliomyelitis yielded slightly positive reactions with 2 to 4 per cent of sera with salt solution extracts of spinal cord, pons and medulla, cerebellum and cerebrum. Tests with extracts of poliomyelitic liver and spleen were uniformly negative, as were all tests employing alcoholic extracts of these different tissues.

5. With polyvalent suspensions of diplococci and streptococci cultivated from persons suffering with acute anterior poliomyelitis, weakly positive reactions were observed with 6 to 9 per cent of cerebrospinal fluids of 35 persons suffering with the disease; with sera all tests were negative, as likewise all tests with fluids and sera employing suspensions of the diphtheroids and Gram-negative bacilli cultivated from poliomyelitis cases. These results indicate that the micrococci of poliomyelitis may produce antibodies during the course of the disease in a manner analogous to the production of antibodies by streptococci in scarlet fever.

6. The Wassermann reaction in acute anterior poliomyelitis is uniformly negative with both cerebrospinal fluid and blood serum.

7. These tests indicate that suitable salt solution extracts of various tissues from fatal cases of acute anterior poliomyelitis may serve in a very small percentage of cases to fix or absorb a small amount of complement in the presence of large amounts of cerebrospinal fluid, but the results in general are in accord with those reported by Wollstein, Gay and Lucas and the reactions are too irregular and weak to be of any practical value in the diagnosis of the disease.

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# SCIENTIFIC PROCEEDINGS OF THE SOCIETY FOR SEROLOGY AND HEMATOLOGY, NEW YORK

February 14, 1917

## 1. THE EFFECT OF INJECTIONS OF TYPHOID BACILLI ON TUBERCULOUS GUINEA-PIGS

*E. S. L'Esperance:* See this issue, p. 283.

## 2. EXPERIMENTAL STUDIES ON LYMPHOCYTES—CYTOTOXIC SERA FOR SMALL THYMUS CELLS

*A. M. Pappenheimer:* The behavior of tonsil and thymus lymphocytes has been studied under a variety of experimental conditions. As a quantitative index of cell injury use was made of the diffuse staining of the nucleus and cytoplasm of the injured cells with dilute solutions of trypan blue. The percentage of stained cells, determined in a blood counting chamber when compared with that of control suspensions maintained under comparable conditions, was found to afford a delicate measure of cell injury.

This technique was applied to a study of the effect of cytotoxic sera for human tonsil and rat thymus lymphocytes. By immunizing rabbits intravenously with washed suspensions of these cells, sera were obtained which when inactivated and reactivated with guinea-pig complement, were definitely cytotoxic, as shown by the increased permeability of the cells to trypan blue. The toxicity of the alien sera was controlled by exposing the cells to equivalent amounts of normal non-immune sera.

The immune sera were strongly agglutinative for the cells used as antigen, the agglutinin being coetostabile.

The thymotoxic and lymphotoxic action of the serum *in vitro* was found to persist after complete absorption of the hemolytic factor.

A hemolytic serum, prepared by immunizing rabbits against washed rat erythrocytes, also exhibited strong agglutinative and cytotoxic properties against rat thymus cells *in vitro*. These also persisted after complete absorption of the hemolysin and hemagglutinin.

A full report of these experiments will appear in the Journal of Experimental Medicine.

## 3. THE ADVANTAGES OF TRIKRESOL IN ANTITOXIC PLASMA AND ITS EFFECTS ON CONCENTRATION

*Edwin J. Banzhaf:* (Did not submit abstract on request.)

#### 4. COMPARATIVE TESTS OF ANTIGENS AND OF INCUBATION TEMPERATURES FOR THE WASSERMANN REACTION

*J. Wheeler Smith, Jr. and W. J. MacNeal:* (1) The use of the cholesterinized antigen; with the first incubation at 8°C. for four hours, constitutes a more sensitive test for syphilis than does any of the other methods examined.

(2) The simple extract antigen, with the first incubation at 8°C., is more sensitive than the cholesterinized antigen at 37°C.

(3) The cholesterinized antigen, both at 37°C. and at 8°C., is apt to yield non-specific complement-fixation. Therefore, in a diagnostic reaction, fixation with the cholesterinized antigen alone is, at best, of only doubtful significance. The simple extract antigen at 8°C. may occasionally give a false positive reaction.

(4) The acetone-insoluble preparation, made according to the method of Noguchi, is less sensitive, either at 37°C. or at 8°C. than is the cholesterin-reinforced antigen at either temperature, and also less sensitive than the simple extract at 8°C. It is more sensitive than the simple extract at 37°C. and in this series has, according to the available evidence, given no false positive reactions.



# ON THE NON-INFLUENCE OF INJECTIONS OF PURE PROTEINS UPON THE PROPORTIONS OF GLOBULIN AND ALBUMIN IN BLOOD SERUM<sup>1</sup>

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A considerable share of the work done by investigators on the chemical nature of immune bodies has confined itself to the study of the proteins of the blood serum. Many determinations of serum proteins have been reported, both of animals and man, in the normal and fasting condition, and during the course of various pathological conditions. Immune bodies (antitoxins, precipitins, agglutinins, etc.) have, to a large extent, become associated with the globulin fraction of the blood serum, since as a rule the globulins were found to increase during the process of immune body production, either on injection of toxin, killed culture, or by infection with a living organism. On fractionation of such immune sera, antibodies have generally been found in the globulin fraction.

Thus, Pfeiffer and Proskauer (1) found that the cholera immune bodies which give rise to Pfeiffer's phenomenon are found in the globulin fraction only. Brodie (2) reported that diphtheria antitoxin belongs to the proteins and is precipitated in the globulin fraction of blood serum. Further work on diphtheria antitoxin was done by Hiss and Atkinson (3), who found that new globulins are formed or rather greatly increased in the sera of horses immunized against diphtheria toxin, and that these globulins served to protect animals inoculated with the toxin. However, Atkinson (4) was unable chemically to differentiate the globulins of normal and antitoxic sera. Pick (5) likewise found that immune bodies were associated with the globulins

<sup>1</sup> Aided by a grant from the George Williams Hooper Foundation for Medical Research.

and not with serum albumin. Seng's (6) results are in accord with those of Hiss and Atkinson and he was likewise unable to determine any difference chemically between the serum of normal and diphtheria immune horses. Ledingham (7) found on immunizing several horses with diphtheria toxin that a horse which failed to produce any amount of antitoxin likewise did not show an increase in the globulin content of the blood serum, whereas in a horse that yielded a high grade of antitoxin the percentage of globulin progressively increased. In a goat the albumin fraction was mainly affected and the globulin fraction to a lesser degree. Banzhaf and Gibson (8) found on fractionating antitoxic sera that the saturated sodium chloride soluble serum globulins of the higher fractions are uniformly much more potent in antitoxin than are those precipitated by lower concentrations of ammonium sulphate. Gibson and Collins (9) state that agglutinins developed in the rabbit, goat and horse, were found in similar amounts in the pseudo- or euglobulin fraction, whereas in horse and goat diphtheria antitoxic serum there was an unequal distribution of antibody content in these fractions. On immunizing horses with diphtheria and tetanus toxins Banzhaf and Gibson (10) found that while there is an increase of globulins in the blood serum, this increase tends relatively to precede instead of paralleling the increase of antitoxin.

Working with antistreptococci serum, Rodhain (11) obtained the antibodies in the euglobulin, but not in the albumin or in the pseudo-globulin fraction. In the sera of infected animals Langstein and Mayer (12) obtained an increase of globulins and a decreased percentage of albumin. Moll (13) carried out extensive investigations on experimental animals, which were given injections of various substances—horse serum, serum globulin, serum albumin, egg albumin, gelatin, salt solution, etc., for an extended period. Nearly all animals showed an increased percentage of globulins. In cases where no increase was obtained, he likewise found no precipitin. The majority of his experimental animals lost weight. He believes that certain substances in blood combine with the immunizing substance to form precipitates which give rise to an increase of globulins. However, he concludes that there is no relationship between the increase of globulins during immunization and loss of weight, since starvation does not prevent precipitin formation. The results of Moll do not entirely point to a direct relation between an increase of globulins on injection of an antigen and immune body formation, since gelatine, which has been shown by Wells (14) to be non-antigenic (it does not sensitize guinea-pigs for

the anaphylaxis reaction) and non-toxic, likewise caused an increase in the serum globulins of the injected animal. Moll (15) also finds, on heating serum albumin in the presence of a small quantity of alkali, that a conversion into serum globulin takes place. On the basis of this work the supposition that the increase of globulins which was observed in the sera of animals during the process of immunization was formed by the conversion of serum albumin into globulin finds no true support. For the conversion of albumin into globulin, glycocoll must be supplied. On heating of albumin in the presence of alkali, such a conversion can not take place. Gibson (16) points out that the "artificial" globulin is merely an intermediate stage in the formation of the alkali metaprotein. From the work of Chick and Martin (17) we can see the probable error of Moll's work. They show that in acid and alkali solutions egg albumin is denatured and the rate is increased with increasing concentration of either  $H^+$  or  $OH^-$  ions. When egg albumin is heated with a slight excess of alkali, no apparent change is noticed. But, on adjusting the solution to the right acidity, the denatured protein is agglutinated in the presence of ammonium sulphate.

Glaessner (18), on carrying out experiments similar to Moll, finds that when animals are immunized with bacteria, toxins, and proteins (horse and beef serum), an increase in the serum globulins can take place, but when animals are carefully immunized by using small quantities of antigen, this globulin increase need not take place, and is not necessary for immune body production, and usually takes place on account of a disturbance in the metabolism of the animal. The results of Müller (19) are essentially in accord with those of Glaessner. He first points out that the blood plasma of normal rabbits is subject to considerable variations with respect to the protein content. On injection of avirulent killed cultures he obtained no considerable increase of the globulins, due to the fact, as he believes, that the injections were made over a short period of time. However, he reports a fibrinogen and total protein increase in his immunized animals. Ruediger (20) finds that streptolysin is precipitated with the globulins and has as yet not been separated from them. He considers that it probably is a globulin.

In the work reported by Quinan (21) there was a distinct departure from the work previously reported. He finds that the substances which cause cytolysis of red blood cells are neither the soluble nor insoluble globulin nor the serum albumin. He regards the substance as being probably a ferment.

Apparently other substances beside those which give rise to immune bodies can cause an increase of the globulins. Thus, Cervello (22) reports that after administering antipyrine to dogs he found an increase of both globulins and of total protein. He regards the phenomenon as one due to altered anabolism. His results are confirmed by Breinl (23), who, however, finds that there is a corresponding decrease of albumins when the globulins increase. He believes that the globulins are formed from albumin by the splitting up of cystein. Evidence is lacking to support this hypothesis.

Robertson's method (24) for the determination of globulin, albumin and non-proteins in small quantities of serum has led a considerable number of investigators (25) to use this method in the study of blood serum obtained under varying conditions. The method enables one to do many determinations in a day, requires small quantities of serum, and gives a fair degree of accuracy. Daily determinations on the serum of animals or man can easily be made and thus the possible effects of experimental conditions followed. Using this method, Tranter and Rowe (26) determined the protein fraction in a large number of normal individuals in order to obtain average values for comparison with values obtained in pathological conditions. Rowe (27) continued this work and extended the investigations to cover a large number of infectious diseases. In all infections, except acute tonsillitis, typhoid and certain mild infections, the globulins were definitely increased. While the globulins were increased in syphilis, apparently there is no direct relation between antibodies which give rise to the Wassermann reaction and those which cause the increase of serum globulins.

Hurwitz and Meyer (28) carried out extensive studies on experimental animals to study the relation of the serum proteins to infection and immunity. Their work is the most extensive and complete so far reported. They find that the progress of an infection is usually associated with an increase of globulins, provided that the infection is severe; a mild chronic infection may continue for a long time without causing a marked change in the relation of the serum proteins. Immunization with carefully controlled doses could be attained without a decided increase of globulins; ordinarily when an animal reacts severely, the globulins rise. This rise may antedate the production of immune bodies. Inflammatory irritants may also cause a rise of serum globulin. In referring to the work of Moll, they have overlooked the serious objections pointed out here and by others elsewhere.

Previously to the publication of the work of Hurwitz and Meyer,

Righetti (29), working in this laboratory, reported the results of his experiments on the effect of the injection of the Gay-Claypole (30) typhoid vaccine on the serum proteins of both normal and typhoid immune animals. He finds that on intravenous injection of a *single* dose of typhoid vaccine into normal rabbits a decided increase of globulin and a corresponding decrease of serum albumin occurs. On re-injecting previously immunized animals, whose globulins percentage is usually higher than that of normal animals, the globulin-protein ratio falls slightly, followed by a considerable rise, primarily a result of the decrease in the percentage of both albumin and total protein present in the serum. The actual amount of globulin present in the serum remains almost unaltered, though the percentage of the globulin in terms of total protein increases.

The present work was primarily a result of the work of Righetti and was largely completed before the publication of work in this field by Hurwitz and Meyer. We have sought to find a possible relation between immune body production and the increase of globulins, which, as has been shown, usually occurs during immunization. If the production of immune bodies be in any way dependent on the increase of globulins, no change should occur on the injection of a non-antigenic substance. Likewise if this phenomenon be due to the toxicity of the injected substance, we should expect a differentiation between a toxic and non-toxic substance. For our work we have chosen to use pure proteins or protein derivatives, since these are definite chemical substances and by working with them the number of variables is reduced. For injecting substances we chose casein, which is antigenic and non-toxic (31); globin, which is non-antigenic and toxic (32); protamine, which is non-antigenic and toxic (33); deuterio-albumose, which is non-antigenic and non-toxic (34), yet complex in composition and glycocoll, which is non-antigenic and non-toxic and very simple in composition.

Our experimental conditions were very much the same as those used by other recent workers in this field. Normal rabbits were kept under constant conditions before and during the experimental period. Blood was taken from the ear vein (opposite to that used for injection); it was centrifuged and the pro-

teins were determined refractometrically. All determinations were made the same day so that there were no chances for decomposition or changes in the serum. The rabbits were fed once daily, directly after bleeding. Enough food was given to last through the day only. In this way the disturbing influences of variable feeding were avoided. The gas burner in the refractometer room was lighted several hours before determinations were made, in order to secure a constant room temperature. All solutions were likewise allowed to come to the room temperature by allowing them to stand near the refractometer a short time. Check determinations showed that there was practically no error in determining non-proteins, and a maximum error of  $\pm 0.2$  per cent in the determination of globulins and albumin. Since our preparations of casein and globin were precipitated from alcohol and ether, they were sterile. They were dissolved in 5 cc. of M/6 salt solution without addition of antiseptic. The casein solution was made neutral to litmus by the addition of 5 cc. of N/10 NaOH per gram of casein. The other substances, protamine, deuterio-albumose and glycoll, were sterilized by heating.

Leucocyte counts were made daily on all of the animals. Blood was taken while the animal was being bled from the ear vein. The counts were made with the aid of the Thoma-Zeiss haemocytometer, a large number of squares in all cases being counted to reduce the error to a minimum. These counts were made to determine whether the injection of a protein would produce a change in the leucocyte count persisting for periods greater than twenty-four hours, and also as a check on the animal's freedom from acute infection. That the injection of a foreign protein body is usually accompanied by a marked change in the leucocyte count is borne out by the experiments of Aschenheim (35) with blood sera, Ledingham's (36) experiments with diphtheria toxin and the more recent work of Gay and Claypole (37) and McWilliams (38). The injection of pure proteins, as is to be expected, produces, as we intend to show elsewhere, a decided change in the leucocyte count. Counts taken at half hour intervals for a period of twelve hours following the injection

of the protein show a leucopenia rapidly followed by a leucocytosis. The magnitude of the rise is not as great as that reported for the injection of bacteria. Our counts at twenty-four hour intervals following the injection of a protein do not show a decided change from the normal.

The experimental results are given in the following tables, the period covered by each experiment, the substance injected, the leucocyte count and the percentages of non-proteins, globulin, albumin, total protein, globulin and albumin calculated as percentage of the total protein, the globulin-albumin ratio, etc., being given in each table.

TABLE 1\*

*Effect of injecting glyocoll intravenously into rabbit 870.♂ Weight 2100 grams.  
Animal gained 110 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	15,200	1.3	4.6	1.9	6.5	71	29	2.4	
2	17,400	1.1	4.9	1.5	6.4	77	23	3.3	
3	17,900	1.3	4.2	2.0	6.2	68	32	2.1	
4	13,800	1.3	3.8	2.1	5.9	64	36	1.8	
Averages		1.2	4.4	1.9	6.3	70	30	2.4	
5	13,700	1.3	4.0	2.2	6.2	65	35	1.8	200 mgm, glyocoll intra- venously
6	12,500	1.3	4.2	2.0	6.2	68	32	2.1	
7	13,800	1.3	4.3	1.9	6.2	69	31	2.3	
8	18,400	1.2	4.3	1.8	6.1	70	30	2.4	
9	10,900	1.2	4.1	1.7	5.8	71	29	2.4	
10	15,500	1.2	3.7	2.1	5.8	64	36	1.8	
11									
12	10,800	1.1	4.6	1.5	6.1	75	25	3.1	
Averages		1.2	4.2	1.9	6.1	69	31	2.3	

\* In the tables the sign ♀ denotes female and ♂ denotes male. The term protein quotient is defined as the ratio of albumin to globulin.

A perusal of the figures presented shows that in normal rabbits the serum proteins are quite variable. The quantity of the globulins (calculated in percentage of the total proteins) in different animals vary from 25 to 40 per cent, with a corresponding variation of the albumin. While the percentages of the two fractions are considerably more constant in any particular rabbit, still the variation is quite large. As far as could be deter-

TABLE 2

*Effect of injecting glyocoll intraperitoneally into rabbit 899. ♀ Weight 3460 grams. Animal lost 60 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN <i>per cent</i>	ALBUMIN <i>per cent</i>	GLOBULINS <i>per cent</i>	TOTAL PROTEIN <i>per cent</i>	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUOTIENT	REMARKS
1	11,000	1.3	4.8	2.3	7.1	68	32	2.1	200 mgm. glyocoll intraperitoneally
2	9,000	1.2	3.7	2.8	6.5	57	43	1.3	
3	11,500	1.3	4.7	2.2	6.9	68	32	2.1	
Averages		1.3	4.4	2.4	6.8	64	36	1.8	
4	10,200	1.3	3.7	2.7	6.4	58	42	1.4	
5	12,100	1.3	4.2	3.0	7.2	58	42	1.4	
6	12,200	1.2	4.1	2.6	6.7	61	39	1.6	
7	10,100	1.4	4.5	2.3	6.8	66	34	2.0	
8	8,600	1.3	3.6	2.5	6.1	59	41	1.4	
9	9,400	1.4	3.9	2.7	6.6	59	41	1.4	
10									
11	9,100	1.2	5.2	2.2	7.4	70	30	2.4	
Averages		1.3	4.2	2.6	6.7	62	38	1.7	

mined, rabbits showing 40 per cent of globulins were free from disease and had previously not been used for experimental purposes. The high figure could not be associated with any lot of animals, nor with those kept in the same cage or order of bleeding. The proteins in individual rabbits were also quite variable though the variability was not as great as that seen between different rabbits. No normal rabbit gave a protein quotient less than 1.3. The total percentage of proteins in all rabbits is



fairly constant as compared with the fractions, and in each individual rabbit we find only small fluctuations. The non-proteins in all animals are practically constant. From the figures it is easily seen that conclusions can not be drawn from small fluctuations based on a single determination during the fore-period nor on the assumption that the protein fractions in all rabbits are constant quantities. Results based on protein

TABLE 3

*Effect of injecting deutoalbumose intravenously into rabbit 873. ♂ Weight 2530 grams. Animal gained 60 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN Q U O - TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	10,400	1.3	4.2	2.0	6.2	68	32	2.1	
2	13,100	1.3	4.6	1.4	6.0	77	23	3.3	
3	15,400	1.3	4.4	1.6	6.0	73	27	2.7	
4	12,700	1.2	4.5	1.4	5.9	76	24	3.2	
Averages		1.3	4.4	1.6	6.0	74	27	2.8	
5	18,400	1.3	4.0	2.0	6.0	67	33	2.0	100 mgm. deutoalbumose intravenously
6	17,100	1.3	4.2	1.8	6.0	70	30	2.3	
7	17,800	1.3	4.8	1.5	6.3	76	24	3.2	
8	22,400	1.3	4.6	1.6	6.2	74	26	2.9	Ear cut in fight.
9	19,300	1.2	4.3	1.6	5.9	73	27	2.7	
10	14,200	1.2	3.7	2.3	6.0	62	38	1.6	
11									
12	16,300	1.2	4.3	2.1	6.4	67	33	2.1	
Averages		1.3	4.3	1.8	6.1	70	30	2.4	

quotients that vary within the limits of 1.3 and 3 must take into careful consideration the normal variability of the animal based on determinations made for a number of days during the fore-period. It is easily seen that quantities which vary in normal animals as widely as the serum proteins can have no connection with an animal's immunity. It seems more reasonable to assume, pending a rigorous proof, that these fluctuations are

metabolic in character, being influenced by room temperature, time, feeding, amount of food eaten, etc. The normal leucocyte counts also show considerable fluctuations in different animals. This variability (within limits) apparently does not indicate the presence of infections nor a variation in immunity or resistance.

Our hypothesis in regard to a differentiation between antigenic and non-antigenic proteins, based on changes in the pro-

TABLE 4

*Effect of injecting deuterioalbumose intraperitoneally into rabbit 897. ♀ Weight 4040 grams. Animal lost 60 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		per cent	per cent	per cent	per cent				
1	10,100	1.3	5.2	1.6	6.8	76	24	3.3	
2	13,300	1.2	5.3	1.6	6.9	77	23	3.3	
3	12,900	1.3	4.6	2.0	6.6	70	30	2.3	
4	11,200	1.3	4.2	2.1	6.3	67	33	2.0	
Averages		1.3	4.8	1.8	6.7	73	28	2.7	
5	14,700	1.2	4.2	2.1	6.3	67	33	2.0	150 mgm. deuterioalbu- mose intraperitoneally
6	11,900	1.3	4.4	2.2	6.6	67	33	2.0	
7	10,300	1.2	4.7	1.8	6.5	72	28	2.6	
8	12,200	1.4	4.7	1.7	6.4	73	27	2.8	
9	10,800	1.2	4.3	2.0	6.3	68	32	2.2	
10	10,600	1.3	4.0	2.0	6.0	67	33	2.0	
11									
12	9,700	1.2	4.6	1.6	6.2	74	26	2.9	
Averages		1.3	4.4	1.9	6.3	70	30	2.4	

tein fractions of the blood serum of rabbits receiving such injections, has received no support from the figures presented. Within the ordinary limits of variation we can find no decided change in the serum proteins of animals receiving injections of the proteins used by us in the doses indicated. The doses were purposely used in amounts not considered excessive, since it is well known that the production of immune bodies is not dependent on excessive amounts of antigen. We have frequently

immunized animals with pure proteins with doses of antigen only half as large as those used in these experiments. The experiment on rabbit 871 (table 9) immunized to casein shows that there is no material difference in the percentages of serum proteins during the fore-period, during the period of immune body formation and during the period subsequent to the animal's attainment of immunity. In no case has the protein

TABLE 5

*Effect of injecting globin intravenously into rabbit 891. ♀ Weight 3290 grams.  
Animal lost 60 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	10,200	1.3	4.0	2.7	6.7	60	40	1.5	100 mgm. globin intra- venously
2	12,000	1.2	5.6	1.6	7.2	78	22	3.5	
3	9,200	1.3	5.0	2.2	7.2	69	31	2.3	
4	9,000	1.3	4.6	2.2	6.8	68	32	2.1	
Averages		1.3	4.8	2.2	7.0	69	31	2.4	
5	9,900	1.2	4.3	2.6	6.9	62	38	1.7	
6	10,500	1.2	4.7	2.2	6.9	62	32	2.1	
7	7,800	1.2	4.6	2.1	6.7	69	31	2.2	
8	10,100	1.3	4.5	2.4	6.9	65	35	1.9	
9	10,200	1.2	4.3	2.3	6.9	67	33	1.9	
10	9,300	1.3	3.9	2.4	6.3	62	38	1.6	
11									
12	7,400	1.2	4.2	2.0	6.2	68	32	2.1	
Averages		1.2	4.4	2.3	6.7	65	34	1.9	

quotient fallen below unity on injection of a foreign proteid substance. This is in accord with previous observations, particularly that of Hurwitz and Meyer, to which reference has already been made, that an increase in the globulin fraction of blood serum is not necessary for the production of immunity. Pure proteins of moderate dosage when introduced parenterally are probably rapidly absorbed, so that the reaction on the part of the animal is of short duration. Egg-albumin apparently seems

to be an exception, the results obtained by Cramer (39) and Porter (40) showing that the parenteral introduction of this substance results in the excretion in the urine of the unchanged substance in considerable amount. On the other hand, when an animal is infected with large quantities of virulent bacteria, there is a continuous reaction on the part of the animal towards the toxins and other bacterial end products. The fact that

TABLE 6

*Effect of injecting globin intraperitoneally into rabbit 872. ♂ Weight 2530 grams.  
Animal gained 110 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	9,800	1.3	4.8	1.3	6.1	79	21	3.7	
2	10,200	1.2	4.9	1.3	6.2	79	21	3.8	
3	9,600	1.2	4.3	1.4	5.7	75	25	3.1	
4	9,300	1.3	4.2	1.6	5.8	72	28	2.6	
Averages		1.3	4.6	1.4	6.0	76	24	3.3	
5	12,300	1.3	4.8	1.2	6.0	80	20	4.0	100 mgm. globin intra- peritoneally
6	11,300	1.4	4.3	1.5	5.8	74	26	2.9	
7	10,700	1.2	4.5	1.5	6.0	75	25	3.0	
8	11,400	1.4	4.7	1.2	5.9	80	20	3.9	
9	10,700	1.3	4.6	1.3	5.9	78	22	3.5	
10	13,800	1.3	4.2	1.8	6.0	70	30	2.3	
11									
12	10,300	1.2	4.2	1.7	5.9	71	29	2.5	
Averages		1.3	4.5	1.5	5.9	75	25	3.2	

antipyretics can produce a change in the protein quotient is another indication of the non-dependence of immunity on globulin increase.

Our results also indicated that there was no apparent parallelism between the leucocyte count and the percentage of serum globulins. It was thought of interest to determine whether this held even in extreme conditions of leucocyte variation. We reversed the usual procedure and by the injection of benzol

produced a condition of extreme leucopenia. The injections were made in the manner and dosage used by Rusk (41). He reports that benzol intoxicated rabbits produce hemolysins and

TABLE 7

*Effect of injecting protamine sulphate intravenously and intraperitoneally into Rabbit 874 B. ♂ Weight 3000 grams. Animal lost 30 grams during experiment*

DAY OF EXPERIMENT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN Q U O - TIENT	REMARKS
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	1.3	3.6	2.3	5.9	61	39	2.6	
2	1.3	3.5	2.5	6.0	58	42	2.4	
3	1.3	4.0	2.0	6.0	67	33	3.0	
4								
5	1.3	4.2	1.8	6.0	70	30	3.3	
6								
7								
8	1.3	3.8	2.4	6.2	61	39	2.6	
9	1.3	4.4	1.9	6.3	70	30	3.3	
Averages	1.3	3.9	2.2	6.1	65	36	2.9	
10	1.3	4.2	2.1	6.3	67	33	3.0	50 mgm. protamine sulphate in- travenously
11	1.3	4.0	1.9	5.9	68	32	3.1	50 mgm. protamine sulphate in- travenously
12	1.3	4.2	2.0	6.2	68	32	3.1	75 mgm. protamine sulphate in- traperitoneally
13	1.4	4.2	2.1	6.3	67	33	3.0	75 mgm. protamine sulphate in- traperitoneally
14								
Averages	1.3	4.2	2.0	6.2	68	33	3.1	
15	1.4	4.5	1.8	6.3	71	29	3.5	
16	1.3	4.2	1.9	6.1	69	31	3.2	
17	1.4	4.2	1.9	6.1	69	31	3.2	
18	1.4	3.8	2.2	6.0	63	37	2.7	
19	1.3	4.0	2.0	6.0	67	33	3.0	
Averages	1.4	4.1	2.0	6.1	68	32	3.1	

precipitins much less efficiently than normal rabbits. Our results (see tables 10 and 11) show that while there is no material change in the protein quotient, a decrease in the percentage of

TABLE 8

*Effect of injecting casein intravenously into rabbit 874. ♂ Weight 2500 grams.  
Animal lost 30 grams during experiment*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	9,800	1.2	4.5	1.7	6.2	73	27	2.7	100 mgm. casein intra- venously
2	9,700	1.3	4.6	1.4	6.0	77	23	3.3	
3	7,200	1.3	4.2	1.7	5.9	71	29	2.5	
4	7,900	1.5	4.5	1.3	5.8	78	22	3.5	
Averages		1.3	4.5	1.5	6.0	75	25	3.0	
5	6,000	1.3	3.5	2.0	5.5	74	36	1.8	
6	8,300	1.3	4.4	1.6	6.0	73	27	2.8	
7	8,100	1.3	4.0	2.0	6.0	67	33	2.0	
8	11,000	1.3	4.1	1.7	5.8	71	29	2.4	
9	8,200	1.2	3.8	2.1	5.9	64	36	1.8	
10	9,300	1.2	3.5	2.3	5.8	60	40	1.5	
11		1.3	3.6	2.2	5.8	62	38	1.6	
12	10,500	1.2	3.9	2.0	5.9	66	34	2.0	
13	11,000	1.2	3.7	2.0	5.7	65	35	1.9	
14	14,700	1.2	4.1	1.9	6.0	68	32	2.2	
15	14,200	1.3	3.8	2.0	5.8	66	34	1.9	
16	11,600	1.2	3.8	2.4	6.2	61	39	1.6	
Averages		1.3	3.9	2.0	5.9	66	34	2.0	

TABLE 9

*Effect of injecting casein intraperitoneally into rabbit 871. ♂ Weight 2840 grams.  
Weight of animal at end of experiment 3460 grams.*

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
1	10,200	1.1	4.8	2.1	6.9	70	30	2.3	100 mgm. casein intra- peritoneally
2	13,000	1.2	4.7	1.8	6.5	72	28	2.6	
3	12,800	1.2	4.3	2.1	6.4	67	33	2.0	
4	15,200	1.3	4.4	1.8	6.2	71	29	2.4	
Averages		1.2	4.6	2.0	6.5	70	30	2.3	
5	13,900	1.3	3.3	2.4	5.7	58	42	1.4	
6	10,000	1.3	4.0	2.0	6.0	67	33	2.0	
7	14,200	1.2	4.5	2.0	6.5	69	31	2.3	
8	11,300	1.3	4.2	2.0	6.2	68	32	2.1	
Averages		1.3	4.0	2.1	6.1	66	35	2.0	

TABLE 9—Continued

DAY OF EXPERIMENT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>				
9	12,900	1.2	3.9	2.2	6.1	64	36	1.8	100 mgm. casein intra- peritoneally
10	11,000	1.3	3.6	2.6	6.2	58	42	1.4	
11		1.2	4.3	2.1	6.4	67	33	2.1	
12	10,900	1.2	4.3	2.3	6.6	65	35	1.9	
Averages		1.2	4.0	2.3	6.3	64	37	1.8	
13	14,100	1.2	4.3	1.9	6.2	69	31	2.3	100 mgm. casein intra- peritoneally
14	12,700	1.3	4.6	1.6	6.2	74	26	2.9	
15	13,500	1.2	3.7	2.3	6.0	62	38	1.6	
16	14,400	1.2	3.8	2.6	6.4	59	41	1.5	
17	14,100	1.3	5.0	1.7	6.7	75	25	2.9	
18									
19	12,000	1.3	3.6	2.8	6.4	56	44	1.3	
20	12,000	1.3	4.2	2.4	6.6	64	36	1.8	
Averages		1.3	4.2	2.2	6.4	66	34	2.0	
21	14,100	1.3	3.6	2.1	5.7	63	37	1.7	100 mgm. casein intra- peritoneally
22	13,100	1.3	5.0	1.5	6.5	77	23	3.3	
23	12,100	1.3	4.4	2.1	6.5	68	32	2.1	
Averages		1.3	4.3	1.9	6.2	69	31	2.4	
24	10,100	1.3	4.2	2.1	6.3	67	33	2.0	100 mgm. casein intra- peritoneally
25									
26	12,100	1.3	4.6	2.3	6.9	67	33	2.0	
27	11,100	1.3	4.4	2.5	6.9	64	36	1.8	
28	11,300	1.3	5.0	1.7	6.7	75	25	3.0	
38		1.3	4.0	2.5	6.5	62	38	1.6	Alexin fixation using 1-500 antigen, positive 0.006 cc. (in terms of serum dilution)
Averages		1.3	4.5	2.3	6.8	67	33	2.1	
39									100 mgm. casein intra- peritoneally
41									100 mgm. casein intra- peritoneally
43									100 mgm. casein intra- peritoneally
56	14,800	1.3	4.2	2.1	6.3	67	33	2.0	
62									Alexin fixation using 1-500 antigen, positive 0.004 cc.

TABLE 10

*Effect of leucopenia (induced by benzol) on the serum proteins of rabbit 848. ♀*  
*Weight 2270 grams. Animal lost 230 grams during experiment*

DAY OF EXPERIMENT	WEIGHT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
	grams		per cent	per cent	per cent	per cent				
1	2270	7500	1.3	4.4	2.5	6.9	64	36	1.8	
2		7000	1.3	3.7	2.9	6.6	56	44	1.3	
3		7500	1.3	4.2	2.2	6.4	66	34	1.9	
Averages		1.3	4.1	2.5	6.6		62	38	1.7	
4		5440	1.3	4.2	3.2	7.4	57	43	1.3	3.5 cc. benzol + 3.5 cc. olive oil subcutane- ously
5		4500	1.3	3.4	2.4	5.8	59	41	1.4	2 cc. benzol + 2 cc. olive oil subcutaneosly
6		2300	1.3	3.4	2.1	5.5	62	38	1.6	1 cc. benzol + 1 cc. olive oil subcutane- ously
7	2270	1120	1.3	3.2	2.4	5.6	57	43	1.3	
8		1400	1.3	3.6	2.3	5.9	61	39	1.6	
9		2720	1.3	3.6	2.4	6.0	60	40	1.5	
10	2160	5740	1.3	3.5	3.0	6.5	54	46	1.2	3 cc. benzol + 2 cc. olive oil subcutane- ously
Averages			1.3	3.6	2.5	6.1	59	41	1.4	
11		3400	1.3	3.2	2.8	6.0	53	47	1.1	Infected with staphylo- coccus
12		1600	1.2	3.5	2.4	5.9	59	41	1.5	
13	2220	1400	1.3	2.3	3.4	5.7	40	60	0.7	
14										
15	2130	1700	1.3	3.4	2.4	5.8	59	41	1.4	
16			1.2	2.8	3.0	5.8	48	52	0.9	
17	2040		1.3	3.2	2.7	5.9	54	46	1.2	Abscess at point of in- fection
Averages			1.3	3.1	2.8	5.9	52	48	1.1	



TABLE II

*Effect of leucopenia (induced by benzol) on the serum proteins of rabbit 843. ♀  
Weight 2950 grams. Animal lost 250 grams during experiment*

DAY OF EXPERIMENT	WEIGHT	LEUCOCYTE COUNT	NON-PROTEIN	ALBUMIN	GLOBULINS	TOTAL PROTEIN	ALBUMIN PER CENT OF TOTAL PROTEIN	GLOBULINS PER CENT OF TOTAL PROTEIN	PROTEIN QUO- TIENT	REMARKS
	grams	per cent	per cent	per cent	per cent					
1	2950	9900	1.3	4.8	1.6	6.4	75	25	3.0	
2		8000	1.4	3.9	2.3	6.2	63	37	1.7	
3		8500	1.2	4.7	1.5	6.2	76	24	3.1	
Averages			1.3	4.5	1.8	6.3	71	29	2.6	
4		5900	1.3	4.4	2.4	6.8	65	35	1.8	2 cc. benzol and 2 cc. olive oil subcutane- ously
5		4900	1.3	4.0	1.8	5.8	69	31	2.2	3 cc. benzol and 3 cc. olive oil subcutane- ously
6	2950	3900	1.3	4.0	1.7	5.7	70	30	2.4	2 cc. benzol and 2 cc. olive oil subcutane- ously
7		2900	1.2	3.7	1.8	5.5	67	33	2.1	
8		2200	1.3	3.6	1.6	5.2	69	31	2.3	1.5 cc. benzol and 1.5 cc. olive oil subcutane- ously
9		2040	1.3	3.6	1.9	5.5	65	35	1.9	2 cc. benzol and 2 cc. olive oil subcutane- ously
Averages			1.3	3.9	1.9	5.8	68	33	2.1	
10	2870	1900	1.3	3.5	2.1	5.6	63	37	1.7	0.4 cc. suspension killed typhoid bacilli I. V.
11		6000	1.3	3.2	2.2	5.4	59	41	1.5	1 cc. typhoid suspension I. V. Blood taken twelve hours after injection
12		3800	1.2	3.5	2.0	5.5	64	36	1.7	
Averages			1.3	3.4	2.1	5.5	62	38	1.6	
13	2700	1600	1.3	3.3	2.2	5.5	60	40	1.5	Infected with staphylo- coccus
14										
15	2620	1340	1.3	2.1	3.3	5.4	39	61	0.6	
16			1.2	2.8	2.9	5.7	49	51	1.0	
17	2700		1.2	3.0	2.6	5.6	54	46	1.2	Abscess at point of in- jection
Averages			1.3	2.8	2.8	5.6	51	50	1.1	

the proteins in the blood serum has resulted, following the repeated injection of benzol. This is not increased after infection, although a decided change in the protein quotient is noted. The fact that after infection the protein quotient becomes less than unity serves both as a check on our determinations, and also as a further indication of the non-dependence of immunity on an increase of globulins. In benzolized rabbits we apparently have a very unfavorable condition for the production of immune bodies, yet the animals responded to the infection by an increase in the serum globulins. It would appear possible that the total proteins of the blood serum are to a certain extent dependent on the leucocyte count. Whether the decrease in the total quantity of serum proteins in extreme conditions of leucopenia is due to a decreased general metabolism, or whether the serum proteins are to a certain degree dependent on the building and destruction of the leucocytes is not indicated. A rise in the serum proteins of both animals following the first injection of benzol seems to lend some slight support to this latter view.

#### SUMMARY

1. Leucocyte counts and determinations of the serum proteins of rabbits receiving doses of antigenic, non-antigenic, toxic and non-toxic proteins have been made for extended periods. This was likewise done with rabbits injected with benzol to produce a leucopenia.

2. The figures show that while the quantity of serum proteins in normal rabbits is fairly constant the protein ratio varies considerably in different animals and to a somewhat lesser extent in individual rabbits.

3. The injection of proteins in doses used was not followed by any decided change in the protein quotient. A decrease of serum proteins follows the injection of benzol, though no change occurs in the protein quotient.

4. A rise of globulins is not necessary for immune body production. Immunity in rabbits may be produced with well regulated dosage of antigen without giving rise to an increase of globulins.

5. Our results are essentially in accord with recent work on the non-dependence of immunity on the serum proteins, as ordinarily determined.

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# IMMUNOLOGIC STUDIES IN TYPHUS EXANTHEMATICUS<sup>1</sup>

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Before discussing the results of immunologic studies made in typhus exanthematicus, the relationship of the *B. typhi-exanthematici* to this disease will be briefly stated.

Up to the present time, this organism has been recovered from the blood of patients suffering from typhus fever endemic or epidemic in the various parts of the world—the United States, Serbia, Bulgaria, Russia and Mexico. It has also been recovered from the blood and spleen of animals, guinea-pigs and monkeys, injected with infectious typhus-fever blood. It has been isolated from typhus-infected lice. It has not been recovered from numerous nontyphus patients, nor from tissues of normal animals, nor from “normal” (nontyphus) lice.

In regard to positive blood cultures in human and experimental typhus fever in animals, the organism can be recovered during the entire febrile period of the disease. It is also found in greatest numbers during the early stages of the disease, especially the chill, and more often in severe than in mild cases. When taken directly from the original colony, the bacteria may be pathogenic, producing in guinea-pigs a febrile reaction after the usual incubation period of typhus fever. During the height of the illness, the *B. typhi-exanthematici* can be recovered from the blood of the animals. The organism is nonfiltrable, corresponding with the nonfiltrability of typhus-infected blood.

<sup>1</sup>Work done under the tenure of the Eugene Meyer, Jr., and a George Blumenthal, Jr., Fellowships in Pathology.

The results of the immunologic studies in typhus exanthematicus tends further to establish the specificity of the relationship of this organism to the disease, by demonstrating that it acts during the height of the fever as the antigen which stimulates the formation of specific antibodies. In discussing these studies, we shall state the results of the investigations of the individual antibody concerned, and then show the correlation of the findings with the disease.

#### AGGLUTININS

Originally these antibodies were studied in endemic (New York) and epidemic (Balkan) typhus fever. It was found that convalescent (immune) serum from one type agglutinated organisms of another type, for example, endemic typhus immune serum agglutinated the organism recovered from an epidemic case. Subsequently similar cross-agglutination was determined in all the forms of typhus—the American, Balkan, Russian and Mexican, thus adding to the factors establishing the identity of typhus fever in the various parts of the world.

Agglutinins were also found in animals which had recovered from experimental typhus fever, except in the case of the guinea-pig. The variability of this animal in regard to antibody formation will be discussed later. In man and monkeys vaccinated by the repeated injections of the organisms, similar agglutinins, usually in large amount, were found.

In the original work, the agglutinins were studied with reference to the time of their appearance during the course of the disease. It was then found that in 43 typhus cases the agglutinins were found in 92 per cent of the patients after the crisis. They were rarely present during the height of the disease, and on the day of the crisis, 33 per cent of the cases gave positive results. In some cases where it was possible to follow the persistence of these antibodies, they were found to be present five to six months after the crisis. This has been confirmed later by Baehr in Russia. He found that the agglutinins are present infrequently in the early stages of the disease but increase in

amount towards the critical period until the seventh or eighth day after the crisis when the maximum titer is attained. He has found the agglutinins to persist usually for three to four months, but occasionally for one year. This typical immunity curve was also confirmed by Denzer, Husk and myself in Mexico in cases of typhus fever epidemic in that country.

These agglutinins are regarded as specific. Sera from a great number of patients suffering from other diseases were tested at the height of the illness, the crisis and post-critically. They showed no agglutinin for the typhus bacillus—except in a few isolated instances when the titer was 1:20 (very rarely 1:50). The microscopic method was used; readings were made at the end of an hour, and in typhus-immune serum the agglutination has been noted in dilutions of 1:1800. Again, organisms other than typhus bacilli fail to agglutinate in the presence of typhus-immune serum. An exception to this has been noted with a strain of the proteus bacillus. But in these instances, Baehr and Paneth have demonstrated that the agglutination is strongest during the fever and rapidly disappears after the crisis—the inverse of the typical immunity curve seen in the agglutination of typhus bacilli by typhus-immune serum.

This transitory appearance of circulating antibodies during the febrile stage of a disease may be looked upon as a result of the stimulation of preformed antibodies normally found in man against various organisms. It is of no significance in the causal relationship of a bacterium to an infectious disease. It is the persistence of the antibodies which characterizes the causal agent of a disease.

#### COMPLEMENT-FIXING BODIES

Working with the protein of the typhus bacillus as antigen, it was shown in the original studies, that the complement-fixing body formation runs parallel to that of agglutinin. It was first demonstrable, however, somewhat later than the agglutinin and disappeared sooner. The cross-fixation of these bodies likewise helped to prove the inter-relationship of the various geographic types of typhus fever. They were also demonstrable

in man and animals vaccinated by repeated injections of the organism. The occurrence of complement-fixing bodies after experimental typhus fever in monkeys was shown but these bodies were not determined in recovered (immune) guinea-pigs—a similar condition with regard to agglutinins.

The complement-fixing bodies like agglutinins appear during the course of, and during the convalescence from typhus fever in the form of a typical immunity curve. During the height of the fever they were rarely present. At the crisis about 20 per cent of the cases were positive. After the crisis about 73 per cent of the patients had positive reactions.

Complement-fixation tests were made subsequently in another series of 40 cases of endemic (New York) typhus fever. The results of these tests confirm the previous observations. Twenty-eight of the 40 cases were tested with the blood removed from the patient at the height of the disease; of these, 20 were negative. Therefore, during the height of the disease, about 28 per cent of the cases showed complement-fixing bodies. Thirty-six of the 40 cases were tested at varying periods of the post-critical stage; of these, 25 were positive and 11 negative. Therefore, in the post-critical period about 69 per cent (compared with 73 per cent obtained in the original studies) of the cases showed complement-fixing bodies. In 24 of these cases it was possible to make tests both during the height of the disease and again after the crisis. This series shows on analysis the formation of the complement-fixing bodies to correspond to a typical immunity curve. For of these, 5 which were negative at the height of the disease showed + + + + reactions after the crisis (usually six to eight days after the crisis); 5 which were negative at the height showed + + or + + + reactions after the crisis; 2 which showed + + + reactions at the height of the disease showed + + + + reactions after the crisis and 3 were positive (+ + + +) both before and after the crisis. Of the last 5 mentioned, i.e., of those in which the reactions were positive during the height of the disease, the blood was withdrawn usually one or two days before the crisis. One case was weakly positive (+ to + +) before and after the crisis, while 8 were negative.



During the time these more recent tests were made, 35 specimens of blood from other sources than typhus fever patients were examined. The blood was usually obtained from febrile conditions, such as typhoid fever, pneumonia, influenza, tuberculosis, etc., and was tested at the height of the fever and in the afebrile period. The results were uniformly negative.

#### PRECIPITINS

Precipitin tests were made in the original studies using a polyvalent precipitogen of typhus bacilli. The precipitin reaction corresponded in its curve of formation to that of agglutinin and complement-fixing body. In 10 cases studied at the height of the disease, none was positive; in 19 cases tested in the post-critical period, about 73 per cent were positive. The usual reactions showed precipitation in a dilution of 1:100, although in 3 cases, 1:1500 dilution was positive. In 8 cases, the opportunity presented itself to study the serum withdrawn at the height of the disease and again after the crisis. Of these, 2 were uniformly negative; 3 were negative at the height of the disease and positive in a dilution of 1:100 after the crisis (usually about the sixth day); 2 were negative at the height but positive after the crisis in a dilution of 1:1000, and 1 was negative at the height and positive in a dilution of 1:1500 after the crisis. Thus we see a correspondence of this with the other humoral antibodies.

#### BACTERIOTROPINS

Immune opsonins were determined not only in the serum from typhus fever but also in animals immunized by repeated injections of the bacilli. Although guinea-pigs which had recovered from typhus fever possessed none of the other humoral antibodies, these bacteriotropic substances could be regularly determined in their serum. In human cases of typhus, the immune opsonins were found to increase at the crisis and remain high during the convalescent stage. In this respect they agreed in formation with the other antibodies described. Furthermore,

it was noted that those bacteria which were ingested by the polymorphonuclears of typhus immune subjects were quite readily fragmented and lost their regularity of staining.

#### ANTIBODIES DEMONSTRABLE BY THE DALE METHOD

This method, involving the anaphylactic response, depends upon the muscular contraction of the uterus of a sensitized guinea-pig when brought in contact with the antigen. It was first successfully applied to the study of infectious diseases by Weil. Denzer and I have been able, by this method, to show that guinea-pigs which had recovered from an attack of typhus fever showed in their cells antibodies against the typhus bacillus. Also in man, the serum from typhus fever patients after the crisis shows similar antibodies. They were not present during the height of the disease—thus being parallel to the other antibodies in their formation or absorption by the cells. Furthermore, these antibodies were shown to be specific: there were no similar reactions obtained with the serum of normal individuals or those suffering from other infections and typhus-immune guinea-pigs showed antibodies only to the *B. typhi-exanthematici* and not to the *M. aureus*, *B. typhosus* or *B. acne*.

Therefore the specific antibodies to the typhus bacillus, agglutinins, complement-fixing bodies, precipitins, immune opsonins and “anaphylactic” antibodies—their formation corresponding with an immunity curve—have been demonstrated in typhus exanthematicus. Recently Sellards has attempted to show that the complement-fixing bodies were nonspecific. He also claims to have isolated from several spleens of pernicious anemia an organism which morphologically is somewhat similar to the Plotz bacillus. In regard to Sellards’ observations, we may state that we have not been able to confirm them. He bases the identification of his organism upon its cultural and serological characteristics. Although a large variety of media was used in his attempts to recover the organism from spleens, he was only successful in isolating his bacillus in litmus milk incubated anaerobically. Milk has rarely been regarded as an optimum

medium, the typhus bacillus grows very poorly in it. The typical picture he describes, namely, acidification of the milk without clotting and precipitation of the proteins, although produced by both the Sellards and the Plotz organisms, may be obtained also by the addition of simple mineral acids. We have seen the same picture in a spleen milk culture, but the organism recovered was a large spore-bearing, Gram-positive, aerobic and facultative anaerobic bacillus.

Sellards has also been unable to grow his organism directly from spleens in much more favorable media, such as ascitic fluid glucose agar or other serum media. In subsequent subcultures he succeeded in growing his organism in ascitic fluid glucose media but it grew better when only small amounts of ascitic fluid were used and its growth was inhibited by the presence of large amounts such as we use. This is exactly contrary to the cultural characteristics of the typhus bacillus. In sugar fermentation reactions his organism also differed materially from the *Bacillus typhi-exanthematici*.

Sellards is influenced in regarding his organism as identical with ours by his serologic results. These are open to criticism. In the first place he does not employ the approved serologic technic and that which was detailed in previous publications. In the second place, his main reliance is based upon cross-fixation between both his and our organisms by the use of rabbit-immune serum. It has been demonstrated by Bernstein, Denzer and myself that this method of classifying organisms leads often to erroneous conclusions. This is mainly due to the fact that such rabbit immune serum contains besides the specific antibodies, antibodies developed to the protein employed in the medium upon which the bacteria in the antigen are grown. The protein antibodies give rise to nonspecific agglutinins and complement-fixing bodies to dissimilar bacteria grown on media containing the same protein. Furthermore, the rabbit is a very sensitive animal, as pointed out by Römer, and Kritschewsky, Kolmer, Huddleson and others advise caution in interpreting complement-fixation results in regard to bacterial antigens.

It has been impossible for us to obtain a culture of Sellards' organism for comparative study.

It is not possible here to offer a categorical reply to Sellards showing other differences between his and our organism except to state our own experience. Up to the present time, four human and six guinea-pig spleens were cultured. Of the human spleens, 2 were removed from cases of pernicious anemia, 1 from acute lymphatic leukemia and 1 from a case of lymphoid hyperplasia (splenomegaly). These were cultured according to Sellards' technic as well as other anaerobic methods. In none of these was there isolated an organism resembling the typhus bacillus. Of the 6 guinea-pig spleen cultures, 4 were sterile, 1 showed the *M. albus* and the other a large Gram-negative unidentified saprophyte. Of the 4 human spleen cultures, the ordinary contaminants, as the *M. albus*, was usually found in 1 or 2 tubes of the culture; otherwise there were no remarkable results.

The correlation of the formation of the antibodies detailed above to the disease shows that they are formed in response to the presence of an antigen, the typhus bacillus, which circulated in the host during the height of typhus fever. Furthermore, the specific nature of the antibodies and the antigen shows the causal relationship of the typhus bacillus to typhus fever. The relationship of antigen to antibody is also brought out in these studies. The antibodies are rarely present during the height of the disease but are to be found uniformly after the crisis. However, in a few cases, antigen and antibody may be found circulating at the same time, i.e., the blood culture may be positive and the complement-fixation reaction may show + + + +. Plotz has also found, in 2 cases, one positive blood culture twelve hours, another positive thirty-six hours after the crisis. In analyzing these facts as well as the fact that an antibody reaction may be negative early after the crisis and positive later (usually on the sixth day) the recent work of Weil, Denzer and others must be taken in consideration. Weil offers the suggestion as a result of work on anaphylaxis and on pneumonia that antigen and antibody may be present simultaneously but the antigen may

interfere with the detection of the antibody. Hence during the course of the disease, in the earliest stages (as in the initial chill, as shown by Baehr and Plotz) enormous numbers of organisms may be present in the circulation; during the height and at the crisis, the number diminishes as the antibodies develop; after the crisis, when there is no trace of antigen left and there is no antigen-antibody combination, the antibodies become demonstrable (a process requiring several days, even up to twelve, for its completion).

At this point it may be of interest to note the development of antibodies in persons who have not had the disease but have been exposed to infection. In the original studies reports of 10 such contacts were given. Subsequently Baehr was able to follow other cases in detail and bring the total number up to 24. In these cases, the contact with the infectious virus consisted in the prolonged handling of virulent blood from patients or animals, the repeated bites of infected lice, or close association with the disease, as in the case of members of the family in which infection occurred. The development of antibodies in these cases, depends upon the principle of immunization by means of inoculation with subliminal infecting doses, as was pointed out by Baehr. The analogy to this development of antibodies in typhus fever may be seen in other infections. As Theobald Smith and others have emphasized, in the presence of epidemics, immunity may follow the development of the mildest type of infection—so mild as to present no clinical picture or symptoms of the disease. However, in view of the fact that so many non-typhus cases show no antibodies and that occasionally antibodies to the *B. typhi-exanthematici* develop in persons exposed to typhus fever, the relationship between the bacillus to the disease becomes obvious.

Another interesting feature of the immunologic studies is the relationship of the guinea-pig to the virus of typhus fever and the production of antibodies. It was stated previously that with the exception of bacteriotropic substances, no circulating antibodies could be demonstrated in this animal. However, by means of the Dale method, it was shown that there is a consid-

erable antibody content in the cells of this animal. The immunity of the guinea-pig which is permanent and of regular occurrence, is then a matter of cellular rather than circulating antibody.

The guinea-pig is the most susceptible animal to typhus fever. An injection of infectious blood in sufficient amount is always followed by typhus fever. A subsequent immunity is regularly developed. But repeated injections of an active virus in amounts insufficient to produce the typical clinical picture of typhus fever does not result in the production of immunity. It is therefore not surprising that our attempts to immunize guinea-pigs by vaccination with dead or avirulent bacteria have been thus far unsuccessful. The ideal method of immunization is to use as antigen, bacteria as closely related to their virulent state (as they circulate in the host during the infection they cause) as possible. In order to develop a definite immunity, the host must react with his defensive mechanism against the introduction of the antigen in a manner similar to that produced by the disease. Thus far we have been unable to obtain strains of sufficient virulence for the completion of this series of experiments.

The basic principle of prophylactic vaccination as it is ordinarily carried out with an avirulent antigen is the increase of resistance to infection, rather than the development of a high degree of immunity. In regard to typhoid fever, as an example, it occasionally happens that a vaccinated person may develop the disease, even though the Widal reaction has been positive as a result of the prophylactic treatment. This is more likely to occur in the presence of a "massive" infection. These factors apply as well to typhus fever. Typhus infection has occurred (as in case of monkeys) even in the presence of agglutinins and complement-fixing bodies. It is generally conceded now that the presence of circulating antibodies is a phenomenon which accompanies the state of immunity; on the other hand, by themselves they do not indicate immunity, as in the case of typhoid occurring while the Widal reaction is positive or in the form of a relapse during convalescence.

Although animal experimentation in regard to the protective action of the injections of avirulent typhus bacilli was unsatisfactory, attempts were made to study the effect in man. This was regarded as justifiable in view of the fact that the injections were harmless, that the great mass of evidence warranted its use and that there was an analogous condition in typhoid fever in which the practicable results by far outweighed those of animal experimentation.

As in typhoid fever, again, prophylactic vaccination with avirulent typhus bacilli has shown that it is capable of reducing the incidence of typhus fever although it does not produce an absolute immunity to the disease. Reports from Eastern Europe by Plotz, Olitsky and Baehr show that out of 8420 persons vaccinated (mainly at the request of the military authorities) 6 developed the disease. Among the vaccinated persons were included only those who came into contact with typhus fever and who were most exposed to the danger of infection. Reports from Mexico of the results of vaccination by Olitsky, Denzer and Husk, of at least 1500 persons, while incomplete, are favorable. Two of the members of the expedition although vaccinated became infected. Their disease may have been caused by "massive" infection, decreased resistance due to the hardships of the environment, and too short an interval between vaccination and exposure. Further reports on all these vaccination studies will be published later.

The immunologic studies add to the proof established by bacteriologic and animal investigations of the causal relationship of *B. typhi-exanthematici* to typhus fever.





# EXPERIMENTS WITH A POSSIBLE BEARING UPON THE SO-CALLED NON-SPECIFIC, INTRAVENOUS VACCINE THERAPY

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The experimental foundation for a non-specific vaccine therapy was laid by Emmerich (1) thirty years ago. He showed that rabbits inoculated with streptococci possessed a certain amount of immunity against anthrax. He also observed that an anthrax infection in rabbits, even after it had called forth distinct symptoms, could be favorably influenced by the injection of streptococci—particularly if the latter injection was administered intravenously. Pawlovsky (2) confirmed these results, and demonstrated that Friedländer's bacillus and *B. prodigiosus* also retarded the development of the anthrax infection. Bouchard (3) found that *B. pyocyaneus* could be used for this purpose. Then Freudenreich (4) and Woodhead and Cartwright Wood (5) obtained similar results with killed cultures of *B. pyocyaneus*, and Buchner (6), with killed cultures of Friedländer's bacillus. Von Dungern (7) injected killed cultures of Friedländer's bacillus intravenously and observed that rabbits so treated were more resistant to anthrax bacilli inoculated subcutaneously one or several days later, than were normal rabbits.

Ichikawa (8), in describing his striking cure by crisis of typhoid fever patients through the intravenous administration of typhoid vaccine noted that paratyphoid patients also were cured by the typhoid vaccine. Kraus (9) showed that *B. coli* vaccine and cholera vaccine may cause the same rapid recovery of typhoid patients as the typhoid vaccine and claimed that the phenomenon, whatever its nature may be, does not represent a specific

reaction. Lüdke (10) obtained similar results by injecting deutero-albumose intravenously into typhoid patients. The observations of these authors have been confirmed by a number of other men. Culver has recently reported the cure of gonorrheal arthritis and epididymitis by means of vaccine administered intravenously. He obtained equally good results with gonococcus vaccine, meningococcus vaccine, and *B. coli* vaccine.

In previous articles (11, 12) we pointed out that the various hypotheses suggested in explanation of the non-specific intravenous vaccine therapy of typhoid fever were unsatisfactory and described some experiments in rabbits which seemed to involve a similar phenomenon. We showed that a rabbit becomes more resistant to infection with typhoid bacilli within twenty-four hours after the intravenous injection of typhoid vaccine. One hundred million killed typhoid bacilli were injected intravenously into a rabbit weighing about 2000 grams, and on the following day this rabbit would survive a large intravenous dose (about 12,000,000,000) of living typhoid bacilli, which dose would kill the control untreated rabbit in twenty-four or forty-eight hours. The following experiments were planned to determine whether this reaction is specific; i.e., whether the protection follows the injection of typhoid vaccine only, or whether it results also from the injection of vaccine prepared from an unrelated organism. For this purpose a strain of *B. coli communis* that had been carried on artificial culture media for more than three years was selected and a suspension of the bacilli in saline was heated one-half hour at 60°C. Since this vaccine was more toxic than our typhoid vaccine, smaller doses were employed. The living typhoid bacilli were injected in every instance twenty-four hours after the preliminary injection of killed *B. coli*. Both injections were made into the ear vein of the rabbit. The results of such experiments are recorded in table 1.

It is seen from table 1 that in seven of the ten experiments the treated rabbits survived, while the controls died in from three to twenty hours after the injection of the living typhoid bacilli. In Experiment II also there is observed a distinctly favorable influence from the injection of *B. coli*; the treated rabbit, in spite

TABLE I

EXPERIMENT	RABBIT	WEIGHT	DOSAGE FOR PRELIMINARY INJECTION OF HEATED B. COLI	DOSE OF LIVING TYPHOID BACILLI	DIED AFTER	REMARKS
		<i>gms.</i>				
I	80	2130	Control	Large dose	50 hours	Killed after nine months
	81	2060	50,000,000	Large dose	Survived	
II	53	2660	Control	15,000,000,000	3 days	Blood, bile, liver and spleen positive
	50	2010	50,000,000	15,000,000,000	6 days	Blood, bile, liver and spleen positive
III	51	1620	Control	15,000,000,000	6 hours	Killed after twenty-four days. Blood, bile, liver, spleen and kidney negative
	48	1435	25,000,000	15,000,000,000	Survived	
IV	89	1880	Control	10,000,000,000	24 hours	
	90	1730	50,000,000	10,000,000,000	48 hours	
V-A	327	1960	Control	10,000,000,000	8 hours	Killed after twenty-one days. Blood, liver, spleen, kidney negative. Bile positive
	326	1960	50,000,000	10,000,000,000	Survived	
V-B	91	2040	Control	10,000,000,000	3 hours	Killed after twenty-one days. Blood, liver, spleen, kidney negative. Bile positive
	330	1880	25,000,000	10,000,000,000	Survived	
VI	331	1820	Control	8,000,000,000	3 days	Bile, spleen, liver and kidney negative. (Intercurrent infection?)
	328	1790	25,000,000	8,000,000,000	15 days	
VII-A	305	2330	Control	12,000,000,000	Survived	Killed after twenty days. Blood, liver, spleen and kidney negative. Bile positive
	322	2220	50,000,000	12,000,000,000	Survived	Killed after twenty-one days. Blood, bile, liver, spleen and kidney negative
VII-B	323	2135	Control	12,000,000,000	20 hours	Killed after twenty-one days. Blood, liver, spleen and kidney negative. Bile positive
	325	2050	50,000,000	12,000,000,000	Survived	
VIII	13	2595	Control	15,000,000,000	6 hours	Killed after twenty-one days. Blood, liver, spleen and kidney negative
	17	2470	75,000,000	15,000,000,000	Survived	

of being 650 grams lighter than the control, lived twice as long. Experiment IV yielded negative results probably because the dose of *B. coli* (50,000,000) was too large for a rabbit of 1730 grams. The four rabbits used in Experiment V-A and V-B all received an injection of the same suspension of typhoid bacilli, as did also those of Experiments VII-A and VII-B.

It is thus demonstrated very clearly that an injection of killed *B. coli* intravenously in rabbits renders them twenty-four hours later less susceptible to an intravenous injection of living typhoid bacilli than are normal rabbits.

Leucocyte counts were made of the rabbits' blood in Experiment VI and are recorded in table 2. At the time of the injection

TABLE 2

	RABBIT 328, TREATED	RABBIT 331, CONTROL
	<i>Leucocytes per</i> <i>cc. mm.</i>	<i>Leucocytes per</i> <i>cc. mm.</i>
Before preliminary injection.....	6,000	9,750
Before test injection.....	8,950	10,000
3 hours after test injection.....	950	800
4 hours after test injection.....	1,450	1,200
25 hours after test injection.....	19,750	13,550
26 hours after test injection.....	23,750	21,950
27 hours after test injection.....	21,850	23,200
28 hours after test injection.....	28,000	22,200
29 hours after test injection.....	22,000	23,800
48 hours after test injection.....	10,600	13,700

tion of the living typhoid bacilli the treated rabbit showed only a very slight increase in the number of leucocytes that could be attributed to the preliminary injection of *B. coli* vaccine; during the succeeding twenty-four to thirty hours both rabbits exhibited a marked leucocytosis. Since the injection of the typhoid bacilli caused a quite similar reaction with regard to the number of leucocytes in the peripheral blood in both rabbits, it would seem that the leucocytosis does not play a part in bringing about the recovery of the treated rabbit.

The results of plating the blood at short intervals after the injection of the typhoid bacilli are recorded in table 3. Six

drops of blood from the ear vein of the rabbit were drawn into 2 cc. of bile or bile plus broth and 1 cc. of the mixture was plated immediately in nutrient agar. The colonies were counted after forty-eight hours' incubation of the plates. The figures in the table indicate, therefore, the number of viable typhoid bacilli in three drops of blood at the different intervals after the injection of the typhoid bacilli. The dosage of typhoid bacilli injected and the weights of the rabbits in the different experiments have been already recorded in table 1. It is seen that there is a

TABLE 3

EXPERIMENT	RABBIT	ONE-QUARTER HOUR	ONE-HALF HOUR	ONE HOUR	TWO HOURS	THREE HOURS	FOUR HOURS	FIVE HOURS	NINE HOURS	TWENTY-FOUR HOURS
III	51 Control...		159	66	24	26				
	48 <i>B. coli</i> ...		250	10	48	3	24			
IV	89 Control...	1,128	376	544	806	656	223	88	24	
	90 <i>B. coli</i> ...	2,800	1088	392	2400	416	122	114	25	
V-A	327 Control...		360	154	700	117	28	36		
	326 <i>B. coli</i> ...		530	97	101	18	4	2	2	6
V-B	91 Control...	330	46	24	600					
	330 <i>B. coli</i> ...	5,000	160	253	800	83	29	3		4
VI	331 Control...	1,540	74	74	363	800	253	138	61	18
	328 <i>B. coli</i> ...	8,000	1060	151	340	86	119	117		20
VII-A	305 Control...	2,040	230	119	102	243	136	70	84	14
	322 <i>B. coli</i> ...	32,000	700	175	1150	332	47	38	153	41
VII-B	323 Control...	4,600	154	1020	604	5140	1490	732	222	
	325 <i>B. coli</i> ...	33,000	1190	137	1170	320	24	17	51	23
VIII	13 Control...	26,800	320	92	1930	2480	752	400		
	17 <i>B. coli</i> ...	41,800	5120	96	544	280	18	18		174

very rapid decrease in the number of typhoid bacilli in the blood during the first hour after the injection and that this decrease is much more rapid in the control rabbit than in the treated one. There is then usually a slight secondary rise at the second or third hour after the injection, but this is of short duration.

Rabbit serum is bactericidal for typhoid bacilli. There is a rapid decline in the number of typhoid bacilli in the blood following the injection of living typhoid bacilli and, if the rabbit lives several days, the bacilli remain in the blood in small num-

bers only, and these probably represent a continual overflow from the organs in which they are proliferating. We (13) have suggested the hypothesis that the intravenous injection of vaccine brings about a transfer of bactericidal substances (complement, or bactericidal antibody, or both) from the blood to the tissue fluids or lymph and in this way increases the resistance of the rabbit. According to this hypothesis the reaction should not be specific; *B. coli* vaccine, as well as typhoid vaccine should be able so to affect the blood capillaries as to allow the passage of these substances through their walls. The above experiments have demonstrated that the reaction is, in fact, non-specific in this sense.

The fact that our rabbits are rendered more resistant to typhoid by a preliminary injection of *B. coli* vaccine as well as by typhoid vaccine and that typhoid patients are cured by crisis by both kinds of vaccine makes it seem all the more likely that the same phenomenon is concerned in both reactions. We believe that our hypothesis offers the best explanation, not only of the new treatment of typhoid fever, but also of those other instances of so-called non-specific vaccine therapy mentioned above; the bactericidal antibodies in each instance are probably transferred from the blood, where they are present in excess, to the tissue fluids where they are urgently needed. It is probable that opsonins and other antibodies, and perhaps complement, are similarly transferred and play a part in destroying the infecting organism in the diseased tissues. The cure would be brought about then, after all, by means of specific antibodies and our ideas with regard to immunity reactions would not have to undergo a radical revision as has been predicted by some writers.

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# SPONTANEOUS AGGLUTINATION IN TYPHOID AND PARATYPHOID CULTURES AND ITS BEARING UPON ABSORPTION OF AGGLUTININS

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In carrying out some experiments involving the injection of living typhoid bacilli in rabbits and the plating several hours later of the rabbits' blood in nutrient agar, we observed that some of the colonies developing upon the plates showed spontaneous agglutination when rubbed up in a drop of saline solution upon a glass slide. A number of the colonies were fished into small tubes of nutrient broth and after twenty-four hours' incubation yielded, instead of the usual turbid growth characteristic of the typhoid bacillus, flocculi throughout the broth and a flocculent sediment. We at first thought that this change in the typhoid strain had been brought about by its sojourn in the body of the rabbit; however, upon plating the original typhoid culture and fishing a number of the colonies into broth we observed a similar type of growth in some of the tubes. We then decided to study the behavior of all of our typhoid and paratyphoid cultures with regard to this phenomenon.

Our stock typhoid cultures were isolated at periods varying from a few months to ten years or more ago. They had been transplanted on nutrient agar at irregular intervals, usually once every three or four weeks and, after twenty-four hours' incubation, were kept in the ice box. The slant agar used in these and the other experiments described in this paper was prepared from meat infusion and was titrated to +1; the plates were poured from nutrient agar made with Liebig's beef extract and titrated to +0.6. Both kinds of agar were prepared and sterilized in

the autoclave. The nutrient broth was prepared from meat infusion and titrated to +1; approximately 1 cc. amounts were sterilized in small test tubes in the autoclave. An American made peptone was used in both the agar and the broth.

The typhoid cultures were transplanted on three successive days for this experiment. A loopful of the twenty-four hours' growth was rubbed up in saline solution and, after appropriate dilution, was streaked over the surface of an agar plate. After twenty-four hours' incubation most of the plates showed several types of colonies; in almost every instance some of the colonies were distinctly more transparent than others. Most of the plates also contained colonies which remained quite small, although well separated from the other colonies. The colonies were for the most part round but occasionally colonies of irregular outline appeared; the latter were usually larger than the former. Twenty colonies of each culture were fished and inoculated into small tubes of broth. It was planned to include all the types of colonies on the plate, but it was impracticable to keep a record of each type of colony fished; the colonies of irregular outline, for example, are included with the round ones in the opaque or transparent group in table 1. Some of the cultures showed a marked difference in the types of colonies, while others allowed a distinction between opaque and transparent colonies to be made only with great difficulty. Colonies of intermediate density were included arbitrarily in one or the other group. The broth tubes were incubated overnight and readings were made in the usual way for macroscopic agglutination.

Table 1 indicates roughly the proportion of transparent, opaque and small colonies on the plate; thus, culture Bel. (+ +, + +, few) had approximately an equal number of transparent and opaque colonies and a few small ones, and culture Gui. (+, + + +) had more opaque colonies than transparent ones. It is seen from table 1 that ten of the twenty-five different typhoid cultures showed complete, or almost complete, spontaneous agglutination in some of the tubes. The bacterial clumps were in some instances large and firm and could be broken up only with

difficulty by shaking; in other instances they were readily disintegrated on being shaken. Some of the cultures showed more or less membrane formation on the surface of the broth. Broth tubes with only a trace of spontaneous agglutination or with a small amount of sedimentation are included in the table in the

TABLE 1

TYPHOID CULTURE	TYPES OF COLONIES ON NUTRIENT AGAR PLATES			SPONTANEOUS AGGLUTINATION IN TWENTY-FOUR HOUR BROTH CULTURE											
	Trans- parent	Opaque	Small	+++			++			+			-		
				T	O	S	T	O	S	T	O	S	T	O	S
76.....		+++	few										1	14	5
Gui.....	+	+++												9	11
G.....	few	+++	few		1		2	13	3					1	
Kal.....	+++	+	few											8	8
Igi.....	few	+++		1	2								3	13	1
Tpf.....		+++			11			2			1			6	
F.....		+++												20	
Car.....		+++	few											15	5
Sal.....		+++												20	
Coy.....	+	+++	+										5	9	6
83.....		+++	few											14	6
Mag.....		+++	few											18	2
Pes.....	few	+++												16	4
Ant.....		+++	few					3	2		9			4	2
79.....		+++	few											17	3
Bel.....	++	++	few										5	12	3
MtS.....		+++	few					13	7						
Pet.....		+++	few					1	1					12	6
L.....	+	+++	few	4							8			3	3
73.....		+++	few											15	5
Pas.....	+	++	few										6	10	4
Stc.....	+	+++											5	15	
Rek.....	few	+++	few	1			4							12	3
Raw.....	+	+++		5	14										
90.....	few	+++			13								5	2	

negative column. The 14 cultures that did not show spontaneous agglutination in any of the broth tubes had been kept under similar conditions as the other eight; hence the changes in the latter should probably be regarded not as induced variations, but rather as true mutations. Since it is generally considered that old cultures show more variants from the type than fresh

ones, the typhoid cultures used in the preceding experiment were kept in the ice box for forty-eight days and were then plated and fished into broth tubes as before; the readings were almost identical with those obtained with the fresh transplants. However, we have since been able to obtain fishings showing spontaneous agglutination in broth from several of the cultures of the negative group.

Our stock cultures of *B. paratyphosus* A, *B. paratyphosus* B and *B. enteritidis* were also plated and fished into broth with the results recorded in table 2.

Eight of eighteen *paratyphoid* A cultures showed complete or almost complete, spontaneous agglutination, as did also sixteen out of twenty-two *paratyphoid* B. cultures and seven out of nine enteritidis cultures. Thus a much higher percentage of these cultures than of the typhoid ones showed spontaneous agglutination in broth. This might be thought to be due to the fact that there are fewer recently isolated strains among them; but the group of typhoid cultures showing spontaneous agglutination contained both old and more recent strains.

Slight clumping or membrane formation is often seen in broth cultures of typhoid bacilli and not infrequently is a source of trouble in performing the Widal reaction. We would like to suggest the use of the above method—plating and fishing a large number of colonies into small tubes of broth—as a possible means of overcoming this difficulty. After twenty-four hours' incubation a tube containing no clumps would be selected and a loopful of material from it transferred to an agar slant, which would then be substituted for the stock agglutination culture.

Complete spontaneous agglutination of typhoid and paratyphoid cultures in broth has been observed before. Kruse, Ritterhaus, Kemp and Metz (1) fished atypical colonies of dysentery bacilli into broth and found that after twenty-four hours' incubation the broth remained clear, while the growth appeared as a heavy sediment composed of clumps of agglutinated bacilli. By using very concentrated peptone-broth they succeeded in cultivating strains of typhoid and paratyphoid that showed a similar behavior.

TABLE 2

CULTURE	TYPES OF COLONIES ON NUTRIENT AGAR PLATES			SPONTANEOUS AGGLUTINATION IN TWENTY-FOUR HOUR BROTH CULTURE											
	Trans- parent	Opaque	Small	+++			++			+			-		
				T	O	S	T	O	S	T	O	S	T	O	S
<i>B. paratyphosus A.</i>															
Dri.....	+++									4			16		
Caf.....	+	+++	few				1			11			5		3
7.....	++	+	few	10	7	3									
60.....		+++	few											18	2
Gro.....	few	+++	few							13	2	4		1	
2.....	+++	few	few		1								10	6	3
4.....	+++		few										17		3
1.....	+++		few										16		4
3.....	+++		few	18		2									
58.....	few	+++	few	4	1		12	3							
62.....	+++												20		
Yal.....		+++	few							17	3				
56.....	+++		few										17		3
And.....		+++											20		
55.....	few	++			7								4	6	3
57.....		++	few										4	13	3
AWB.....	+++		few	9									8		3
Par.....		+++			17	3									
<i>B. paratyphosus B.</i>															
66.....	+	+++	few	5	4	4	2	4	1						
And.....	few	+++	few		4					4	8			1	3
Alg.....	+++	+		1	6								13		
25.....	+++									1			19		
Gac.....	+++	+	few				6			1			8		5
BAm.....		+++		20											
54.....	+++	+	few	1			2						9	3	5
Bos.....		+++	few	11	3		4	2							
63.....		+++	few	15	5										
Sch.....	+++	few	few				1			1			12	3	3
ABo.....	few	+++											10	10	
Whi.....	+++												20		
97.....		+++		6			5			9					
Roa.....	+++	+					1			2			13	4	
IWh.....		+++											20		
Kur.....	+++	+	few	2	1			1		4			8		3
4.....	few	+++	few				8	7	5						
52.....	+++	+		7									13		
Hun.....	+++	few											15	5	
BBe.....	+++	+	few	6						1			10		3
65.....	++	++					10	10							
96.....	+++		+							1			13		6

TABLE 2—Continued

CULTURE	TYPES OF COLONIES ON NUTRIENT AGAR PLATES			SPONTANEOUS AGGLOUTINATION IN TWENTY-FOUR HOUR BROTH CULTURE											
	Trans- parent	Opaque	Small	+++			++			+			—		
				T	O	S	T	O	S	T	O	S	T	O	S
<i>B. enteritidis</i>															
69.....		+++	few										15	5	
Whi.....		+++	few	15	5										
47.....		+++	few			1	15	4							
67.....		+++	few				12	5		2			1		
64.....		+++	few			1							17	2	
70.....		+++	few										15	5	
93.....	++	++	few	7	1	1	7	5							
And.....		+++	few	3	4		3	1					9		
14.....	+++	few	few				3						12	5	

M. A. Barber (2) obtained spontaneous agglutinating cultures without using special culture media. He made his subcultures from single bacilli or filaments which were transferred to broth tubes by means of his own ingenious apparatus involving the use of very fine capillary pipettes. After many such fishings he obtained a race of *B. coli* that grew in long filaments. Cultures of this race in plain broth at room temperature, he says "tend to become flocculent, the flocculi often adhering to the sides of the tubes or settling to the bottom and leaving a comparatively clear liquid in the part of the culture between the pellicle and the sediment. In this respect the new race cultures present a striking contrast with the uniform cloudiness of the check tubes." He observed the new race during a period of two years and eight months and found that its characteristics persisted. He transferred in like manner long filaments of typhoid cultures to broth and found "that variations appear in *B. typhosus*, which, when isolated produce new races similar to those of *B. coli* but of comparatively less stability." The most stable new race of *B. typhosus* that he encountered had reverted to the type fifty-three days after its origin. One of our strains, a subculture of typhoid ninety still shows complete spontaneous agglutination in broth one hundred and sixty-eight days since its isolation.

An attempt has been made recently by Hooker (3) and by Weiss (4) to determine whether various strains of typhoid bacilli belong to one and the same group or to different groups. The former used agglutinin absorption and complement fixation reactions for this purpose; the latter fermentation tests and the agglutinin absorption reaction. Both believed that their strains of typhoid bacilli belong in several different groups. Since their evidence rests largely upon the results of agglutinin absorption experiments, it is timely to point out a source of error that we have observed in such experiments.

We isolated by the above method two subcultures from the same typhoid culture—one agglutinating spontaneously in broth and the other not—and performed absorption experiments with the two against the same serum. We modeled our technique somewhat after the experiments of Weiss. Typhoid immune serum from a rabbit with a titer of 1/16,000 was diluted to 1/2000. To 1 cc. of the diluted serum was added the growth from two agar slants of the agglutinating subculture and to another cubic centimeter, the same amount of growth of the normal subculture. The tubes were shaken, placed in the incubator for three hours, and then centrifugated at high speed for one-half hour. One-half cubic centimeter of a suspension of typhoid bacilli in saline solution was added to 0.5 cc. of each of the supernatant fluids and to a 1 to 1 dilution of each, yielding final dilutions of 1/4000 and 1/8000 of the treated serum. The tubes were incubated overnight and readings were made on the following morning. The results of such experiments carried out with six different typhoid cultures are recorded in table 3.

It is seen from table 3 that the normal subcultures effected the complete removal of the agglutinins from the diluted serum while the spontaneous agglutinating subcultures did not. We could no doubt have obtained similar results with some of our paratyphoid cultures but we purposely chose typhoid cultures for this experiment, because they are considered to be less variable than the paratyphoids.

It would seem, therefore, that the state of physical aggregation, as well as the chemical constitution of bacterial antigens

plays an important part in agglutinin absorption experiments. This is not at all strange when we consider that in preparing the lipid antigen for the Wassermann reaction, we obtain very different results according as we run the alcoholic solution of the lipoids into saline solution slowly or rapidly; the chemical constitution of the lipid antigen is the same in the two instances, but the size of the aggregates in suspension is different. We have selected extreme instances in the experiments recorded in table 3, but it is logical to suppose that cultures showing even

TABLE 3

CULTURES (Incubated three hours in typhoid immune serum diluted 1/2000)		RESULT OF AGGLUTINATION OF TYPHOID CULTURE IGL. WITH SUPERNATANT FLUID AFTER CENTRIFUGATION	
		1/4000	1/8000
L	{ Spontaneous agglutinating subculture....	+++	+++
	{ Normal subculture.....	—	—
G	{ Spontaneous agglutinating subculture....	++	—
	{ Normal subculture.....	—	—
F	{ Spontaneous agglutinating subculture....	++	—
	{ Normal subculture.....	—	—
90	{ Spontaneous agglutinating subculture....	+++	+++
	{ Normal subculture.....	—	+sl
Rek.	{ Spontaneous agglutinating subculture....	+++	++
	{ Normal subculture.....	—	—
Pes.	{ Spontaneous agglutinating subculture....	+	—
	{ Normal subculture.....	—	—

a slight tendency to spontaneous agglutination would absorb agglutinins somewhat less readily than normal cultures. We do not consider that the differences in the size of the aggregates in the bacterial suspensions account for all the differences observed in the complement fixation and absorption experiments of Hooker and of Weiss, but we do think that this factor should be reckoned with in drawing conclusions from such experiments.

Both Hooker and Weiss raise the issue as to whether it is advisable to continue the use of the culture Rawling for the preparation of prophylactic vaccine. Nichols (5) says:



It (the Rawling strain) has been used in the preparation of vaccine in both the English and American armies. The strain was originally obtained from the spleen of a soldier who died of typhoid fever in England in 1900. It was selected originally by Leishman for experimental use in preparing vaccines, not on account of its low toxicity or superior immunizing properties, but because it gave a remarkably even suspension when washed off agar with salt solution.

In view of the remarkably good results obtained with this culture, it will no doubt remain in use as a vaccine until there is a well established experimental basis for a change. Typhoid vaccination is now practiced on such a large scale, that the question here involved is of no mean importance and therefore any peculiarities of the Rawling culture are of particular interest. We obtained our Rawling culture directly from the Army Medical School in Washington through the kindness of Dr. Vedder.

After transplanting the culture a few times on our agar, we plated and fished colonies into small tubes of broth. All twenty of the tubes showed, after twenty-four hours' incubation, a heavy sediment, while the broth above remained clear. Upon shaking, many clumps that were present were readily disintegrated and a homogeneous suspension resulted. The question suggests itself whether the tendency to form clumps may not be a desirable quality in vaccines in that it might lead to a more rapid phagocytosis after the injection. We are not aware that this problem has been approached experimentally as yet.

We also tested the bacteriolytic action of normal rabbit serum upon the Rawling culture and our stock typhoid cultures. We employed the technique described in a previous paper (6) and found that the Rawling strain was more susceptible to the action of the sera than any of the other cultures. The results of these tests are recorded in table 4.

This unusual susceptibility of the Rawling culture to the bacteriolytic action of normal sera may be a factor in determining its suitability for a vaccine.

TABLE 4

EXPERIMENT NUMBER	TYPHOID CULTURE	DOSAGE OF BACILLI PER CUBIC CENTIMETER OF SERUM	NUMBER OF BACILLI SURVIVING IN ONE LOOP OF SERUM AFTER TWENTY-FOUR HOURS INCUBATION	APPROXIMATE NUMBER OF BACILLI KILLED PER CUBIC CENTIMETER OF SERUM
I	L.....	50,000,000	α	
		10,000,000	0	10,000,000
	Pas.....	10,000,000	α	
		1,000,000	3	1,000,000
	Coy.....	10,000,000	α	
		1,000,000	0	1,000,000
		50,000,000	Very numerous	
	90.....	10,000,000	96	
		1,000,000	0	1,000,000
	Car.....	100,000	Numerous	
		10,000	2	10,000
	Sal.....	100,000	α	
		10,000	0	10,000
	83.....	1,000,000	Very numerous	
II		100,000	0	100,000
	Rawling.....	100,000,000	Fairly numerous	
		50,000,000	0	50,000,000
	Rek.....	10,000—	α	10,000—
	G.....	10,000,000	Fairly numerous	
		1,000,000	0	1,000,000
	Kal.....	1,000,000	Fairly numerous	
		100,000	7	100,000
	76.....	1,000,000,	Numerous	
		100,000	7	100,000
	Mt. S.....	10,000,000	Fairly numerous	
		1,000,000	0	1,000,000
	Bel.....	10,000	Fairly numerous	10,000—
	Pet.....	100,000	Fairly numerous	
III		10,000	0	10,000
	Rawling .....	200,000,000	α	
		100,000,000	1	100,000,000
		50,000,000	5	
		10,000,000	0	
	F.....	10,000,000	Fairly numerous	
		1,000,000	0	1,000,000
	79.....	10,000,000	Fairly numerous	
		1,000,000	0	1,000,000
	Igi.....	10,000	Fairly numerous	10,000—
		10,000,000	α	
	Ant.....	1,000,000	0	1,000,000
		200,000,000	37	200,000,000
	Rawling.....	100,000,000	0	

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# THE SELECTION OF HORSES FOR THE PRODUCTION OF DIPHTHERIA ANTITOXIN BY AN INTRA-PALPEBRAL TOXIN TEST

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An interesting result of the work of Park and Zingher<sup>1</sup> upon the immunization of persons with mixtures of diphtheria toxin and antitoxin has been with regard to the production of antitoxin by various individuals. It has been shown that those persons who already possess immunity to diphtheria toxin more readily react to the injections with the production of antitoxin than do those individuals who possess little or no natural immunity to diphtheria toxin. This being the case, it occurred to us that if the same thing held true with horses, we might have, in a modification of the Schick test, some indication of the productive possibilities of horses with regard to diphtheria antitoxin. The experiments that we are about to report indicate that, to a considerable degree, *the antitoxin productiveness of horses can be anticipated with the use of the Schick test.*

It is well known that the most convenient method for making tissue tests in horses and a reaction that gives the most striking pictures is obtained by injecting the test material intrapalpebrally. The intrapalpebral test was selected for these observations.

## TECHNIQUE

The technique employed in making the tests varied somewhat from that employed by Moussu (Recueil. de Med. Veter., July, 1915) in testing cattle with tuberculins.

With a sterile 1 cc. Record syringe, graduated in fifths, fitted

<sup>1</sup> Am. Jr. Pub. Health, 1916, 6, 431.

with a number 25 platinum iridium or a steel needle, 0.2 cc. of diluted diphtheria toxin was injected intrapalpebrally. The dose used in the tests reported has been three minimal lethal doses for a 250 gram guinea-pig. In most cases the toxin was injected as superficially as possible into the conjunctival mucous membranes of the lower lid. Some variations in the technique were made in preliminary tests; one such variation was the injection of the test toxin intradermally just below the palpebral margin of the lower lid.

As a rule the left eye was used for the test, the right eye acting as a control.

#### METHOD OF READING TESTS

Reactions are characterized by the amount of infiltration in the treated palpebrae; the injection of the visible conjunctival membrane; a watery discharge rapidly becoming purulent, and sensitiveness sometimes accompanied by photophobia.

Readings of reactions were made after twenty-four hours and forty-eight hours and the severity indicated as follows:

+ Fullness of the lid into which the injection had been made. Tenderness; marked reddening of the visible conjunctival mucous membrane; and a watery discharge with flakes of pus.

++ Lid swollen so that eye was about half closed. Tenderness; profuse watery discharge and more pus.

+++ Lid badly swollen; eye nearly closed. Painful; photophobia; profuse discharge of a purulent nature.

The reactions as a rule were at their height after about twenty-four hours. Occasionally, however, they appear to increase in severity and sometimes a ++ reaction at twenty-four hours becomes a +++ after forty-eight hours. Earlier or later readings have apparently little value.

In the more severe reactions a slight necrosis of the skin at the point of injection is noticed at the forty-eight hour reading when the injection is made intracutaneously. This necrosis is more plainly evidenced, however, when a later reading is made. The reactions persist as a rule for several days.

Not infrequently a treated eye shows a slight edema of the lid and some injection of the conjunctival mucous membrane on the first observation. But after forty-eight hours the edema and redness have disappeared. Such reactions have been indicated by a =.

These reactions were considered as pseudo reactions and have been classed as negative in the summary.

The results herein reported have been obtained by using a fixed dose of three minimal lethal doses of toxin and so far few attempts have been made to ascertain whether or not a more satisfactory dose might be obtained. The few variations made, however, have not apparently altered the results.

The results of the test applied to sixty-seven previously untreated horses are shown in the following table:

*Summary of results*

HORSES TESTED	REACTIONS	PRODUCTIVE	
		Number	Per cent
15	+	1	6.6
10	++	2	20.0
3	+++	1	33.3
28	Total positive	4	14.3
39	—	29	74.4





## THE RELATION BETWEEN ANTIGEN AND ANTIBODY IN THE LIVING ANIMAL<sup>1</sup>

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It has been said that all sciences pass through three stages, that of philosophic anticipation, that of detailed observation, and that of careful generalization. The science of immunology is now in the second stage, and is engaged in gaining a mastery of the facts upon which reliable generalizations may in the future be based. Knowledge of the details of the science is increasing apace, and day by day the sure foundations are being laid of a permanent and well established theory.

Annually we meet to exchange with one another the fruits of the year's labors, and so to widen and deepen the stream of our knowledge. But, of necessity, we deal with sharply circumscribed problems, because we wish to give one another only such things as we have thoroughly proved and tested. This tacit, but general, law of these meetings was wisely broken last year in Dr. Jobling's presidential address, which aspired to give a broad and critical review of one very important aspect of immunological study. I have decided, though not without trepidation, to emulate this example, and to attempt, in so far as the somewhat narrow confines of our present knowledge will permit, an analysis of the relations of antigen and antibody within the living organism.

The fundamental task of immunology is to investigate the reaction of the living organism to the invasion of foreign material. In the higher organisms, this reaction, so far as we know at present, takes two forms; it manifests itself both in the phagocytic activity of certain special cells, and in the production of certain

<sup>1</sup> Presidential address at the fourth annual meeting of the American Association of Immunologists, held at New York City, April 5 and 6, 1917.

substances known as antibodies. The labors of many years have gone far to elucidate the mode of action of antibodies, so that we may now be said to have a considerable knowledge of this subject. But the studies of earlier years were, of necessity, almost exclusively conducted *in vitro*. Bacteria, for example, were subjected to the action of sera, and from the results observed in the test tube inferences of wide significance were drawn as to the corresponding effects within the body. We have, however, begun to learn that the body does not act precisely like a test tube. Ten years ago, Metchnikoff, in his introductory words in Kraus and Levaditi's Handbuch, made the following pregnant statement:

The guiding principle in the technique of immunological study consists in holding steadily in view the processes which take place in the living organism, and never transferring unreservedly the observations made on test tube reactions to the conditions which obtain in the animal itself.

During recent years there has been a steadily accumulating body of evidence which deals directly with the relations of antigen and antibody *in vivo*, so that there is now little danger that Metchnikoff's wise warning will fall on deaf ears. Owing to the greatly increased difficulties of the problem, however, our knowledge concerning the processes which go on *in vivo* is still far less complete than it is of the test tube reactions. Not alone this, but in one very important respect the two fields of experiment cannot even be compared, and the knowledge gained from the test tube is of no value in understanding the conditions of actual life. This important difference is based upon the fact that in the living animal it is not alone the circulating blood, but the living cells which carry the antibodies. Although the mode of action of the antibodies of serum outside the body affords certain definite indications as to that of the blood within the vessels, there is no possibility whatever at present of studying the reaction of the antibody of the immune cell in the test tube, and of determining its effect upon antigen. Through the intervention of the pharmacologists, however, a new method has

been evolved which permits of the study of the immune cell itself under artificial conditions outside of the body, and thus, even though only indirectly, reveals the relation of cellular antibody to antigen. These are, then, the two main divisions of the problem: first, the reaction of the antibody of the serum, and secondly, that of the antibody of the cells, to antigen.

#### CIRCULATING ANTIBODIES

As regards the antibodies present in the blood, we have been generally accustomed to assume that they exercise the same effects upon the antigenic substance *in vivo* as they do *in vitro*. Nevertheless, this assumption should not be made without reservation, for the reason that the conditions are, in many respects, different. In the one case, these two reagents are in contact in an immobile fluid, whereas in the other they are being hurried pell mell through the vessels, intermingled with the corpuscles of the blood. To anyone who has observed the effects of constant agitation upon the processes of precipitation, or of agglutination, in the test tube, it must be evident that the consummation of such reactions might meet with serious difficulties in the living animal. In addition, there are the phagocytes, both fixed and mobile, in the body, the function of which is to seize upon and remove from the circulation all foreign particles; it seems likely that these cells might rapidly ingest even microscopic aggregates, such as would be offered by minute foci of precipitation or agglutination, and would at once remove them from the plasma. On grounds such as this it has appeared doubtful whether anything comparable with the customary test tube phenomena could take place in the body. Recent observations by Bull indicate nevertheless that such is the case. This author found that pneumococci are agglutinated into clumps and may be demonstrated as such in the heart's blood when injected into immune animals. Admitting this possibility, therefore, there are other considerations which tend to indicate that agglutination *in vivo* is a relatively infrequent occurrence. Typhoid fever is a disease of which the presence of agglutinins is a frequent, almost characteristic accompaniment, neverthe-

less, there is good ground for believing that in this disease agglutination rarely takes place within the body. It is an old and well attested observation that typhoid bacilli freshly recovered from the body are generally inagglutinable in the test tube, which makes it seem very unlikely that any such reaction can occur during the course of the disease. Only after growth in culture for some time do these organisms become agglutinable. Nor does this hold true only of typhoid; Zinsser relates a similar observation of the spirochaete of syphilis. Bull has found that pneumococcus cultures, when first introduced into the vein of a normal dog, are rapidly agglutinated, and disappear from the blood, but that within three days the circulation becomes flooded by a variant of the original strain, which is found to be entirely inagglutinable by normal dog's blood. Eventually, however, the new and resistant strain gives rise to a newly formed agglutinin, which is capable of clumping it in spite of its defensive adaptation. All in all, it is apparent that agglutination plays less of a rôle in the processes of disease than might be assumed *a priori*, judging from the effectiveness of the test tube reaction. As regards precipitation, there are no observations which permit of a conclusion as to whether it actually takes place in life. There are some authorities who seriously doubt this occurrence, and yet it seems difficult to justify this doubt. Soluble proteins, which are the material of precipitation, cannot, of course, undergo adaptive alteration like the bacteria, and become non-precipitable. It is quite likely, however, that microscopic precipitates may be filtered out by the phagocytes as rapidly as formed, and that actual gross flocculation does not, therefore, occur. This would explain the fact that it has never been possible to demonstrate the phenomenon in the circulating blood. On the other hand, it is well known that complement fixation occurs *in vivo* when an antigenic serum is injected into a previously prepared animal, a fact that seems to prove conclusively that precipitation does take place, even though it may not go on to flocculation.

When we admit that agglutinating antibodies may not always exercise this special function *in vivo*, there is no necessary im-

plication that they perform no other useful purpose. Manwaring has recently shown that antibodies which do not agglutinate pneumococci may still alter them in such fashion as to lead to their adhesion to the endothelia of the blood capillaries, which are actively phagocytic cells. One fact is certain, and that is that agglutinins are bound by inagglutinable bacteria with the same avidity as by the agglutinable. Inasmuch as clumping, of itself, does not appear to injure the vitality of bacteria, it is very possible that it is an accidental and inessential function of these antibodies, and that their actual value in the struggle against the latter may be of quite a different kind—of a kind at present possibly unknown to us.

Of the other properties of antibodies as discovered by test tube reactions, there are three of great importance which we know to be duplicated *in vivo*. Lysis, whether of bacteria or of alien red cells, has been familiar as a vital process since the discovery of the Pfeiffer phenomenon, which, indeed, antedates the study of lysis *in vitro*. Intravascular hemolysis of alien red cells in immunized animals is easily demonstrable by the discoloration of the serum of such animals. Complement fixation *in vivo* has been repeatedly demonstrated, notably by Friedberger. Finally, the protective action of antitoxin against toxin is equally manifest whether these substances are mixed *in vitro*, or are separately injected into the animal.

In spite of large gaps in our knowledge concerning the details, it is quite clear, therefore, that a number of highly important processes take place in the circulating blood exactly as they do in the test tube. A very interesting discussion has recently arisen as to the equilibrium of reactions *in vivo* and *in vitro*. Do antigen and antibodies unite to the exhaustion of either factor, or does the reaction proceed to a "dead center" and then stop? This discussion cannot yet be considered to be settled conclusively, but it is important to remember that the conditions which make for a complete reaction are much more favorable in the body than in the test tube. In the former, the products of the reaction are constantly undergoing removal by various special agencies, such as the phagocytes cells, whereas

in the test tube there is no such provision. If, therefore, a law of equilibrium be operative in the test tube experiments, as I however, do not believe that it is, there would still be excellent reasons for thinking that the effects of antibody upon antigen could be much more completely carried out *in vivo*.

#### ANAPHYLATOXIN

One of the most interesting discussions which recent years have brought forth concerns the destruction of antigen by the antibodies of the blood. I refer, of course, to the theory of parenteral digestion of foreign protein. A number of years ago it was shown that when antigen and antibody are mixed and incubated in the test tube, protein disintegration takes place, as evidenced by chemical tests. Coincidentally, it was shown that when a sensitized or an immunized animal is injected with antigen, there is an increase of protein degradation products both in the blood and in the urine. It was naturally assumed that the antigen, when submitted to the ferment-like action of the complement, through the mediation of the antibody, was actually broken down or digested; when this process occurred in the body, it took place, supposedly, in the blood stream and represented what came to be known as "parenteral digestion." This theory was in complete harmony with the well known views associated with the name of Abderhalden. But parenteral digestion was not the last chapter of the story. The products so formed were believed to be highly toxic, in accordance with the observation that peptone, when given intravenously, produces all the symptoms of anaphylaxis in dogs. Thus originated the conception that the parental digestion of foreign protein gives rise to intermediate products which are responsible for the toxic manifestations of anaphylactic shock and of infectious disease. To these products Friedberger gave the collective name of anaphylatoxin, and Vaughan identified them with the toxic fraction which he had been able to split off from protein by chemical procedures.

The progress of research, however, has not tended to confirm any of these views. In the first place, it has been shown that

when antigen and antibody react *in vitro*, it is not the antigen at all, but the antiserum which undergoes chemical alteration. As a matter of fact, the antiserum digests itself—undergoes autolysis. Moreover, this process is not an immune reaction in any sense, inasmuch as it takes place when normal serum (that of the guinea-pig being used) is mixed with an alien normal serum, or even when normal guinea-pig serum is mixed with agar or with starch. The question arises whether anything similar to this takes place when antigen is injected into a sensitized animal. It appears safe to answer this question with an emphatic negative. When a normal guinea-pig is injected with antigen and antiserum, given simultaneously into opposite jugulars, the conditions are entirely analogous to those of the test tube experiment, yet the animal fails to show any symptoms. Evidently anaphylatoxin has failed to make its appearance. Again, if horse serum is injected intravenously into a normal guinea-pig, no effect is produced. Thus one is driven to the conclusion that serum in the test tube reacts quite differently from plasma in the blood vessels, a view which is borne out by the fact that homologous serum of itself may be highly toxic to a guinea-pig. Jobling has shown, however, that in dogs the blood gives definite chemical evidence of protein disintegration in anaphylactic shock. Here again, however, transfusion experiments have revealed the fact that these chemical changes are without influence upon the development of the shock. They are simply the harmless by-products of the anaphylactic reaction of the sensitized liver, and, as a matter of fact, accompany a variety of pharmacologic procedures which injure the liver, such as chloroform or phosphorus poisoning.

Thus there remains no experimental evidence that affords any support whatever to this very interesting theory. Parenteral digestion of the antigen in all probability never takes place in the blood; but if it does, the products are of no influence in the development of the symptoms of anaphylaxis. As a matter of fact, all the evidence is conclusively in favor of the belief that anaphylaxis in the guinea-pig is exclusively a cellular reaction, in which the blood plays no part. Guinea-pigs may, indeed, be

killed by the intravenous injection of a variety of substances, both organic colloids and inorganic substances in solution, but these reactions are in no way related to true anaphylaxis, and throw no light on its mechanism.

#### CELLULAR ANTIBODIES

When we come to the study of cellular antibody and its mode of action, we are on a plane which presents no extra-vital analogy. The cells must be studied as living entities, and although the mode of action of antibodies in solution may afford valuable hints, it can never be considered as yielding definite information concerning the properties of those same substances when a part of the cell. As a matter of fact, we know that the cell materially modifies antibodies when it appropriates them. Thus it has been demonstrated that the avidity of antibody for antigen is very greatly intensified when anchored by the living cell, and it has been shown that this alteration is not accomplished suddenly or rapidly, but that it requires several hours for its maturation. The study of the properties of cellular antibodies has been made possible for us by the discovery of the anaphylactic reaction, which during the last few years has been actively applied to the solution of some of these problems. But it must be remembered that this method is essentially an indirect method. It does not permit of the direct observation of the reactions which take place between antigen and antibody, but it discloses these relationships only by inference through the change which they produce in the cell itself, and through the modifications of cellular function. Notwithstanding the difficulties, and possibly the dangers, of such a mode of study, it has yielded data which are new and important in the study of immunology.

When foreign protein in soluble form finds its way into the circulation, the organism almost invariably makes an effort to get rid of it. Such a purpose might perhaps be most easily and rapidly accomplished by the mere extrusion, or excretion of the alien material. If a dye, such as congo red, is introduced into the circulation, it very slowly makes its exit from the body, at



least for the most part, by way of the bile and of the urine. It might be expected that egg albumin, or horse serum, when parenterally introduced, would be similarly disposed of. Such however, is not at all the case. Even if considerable amounts are introduced, it is, as a rule, impossible to demonstrate the foreign material, even in traces, in the urine. In human beings that had received as much as 100 cc. of therapeutic horse serum intraspinally, I could detect no horse serum in the urine with the help of a very powerful precipitating serum derived from an immunized rabbit. On the other hand, substantial amounts of horse serum were discovered in the circulating blood of this same individual, by the same reagent, for more than three weeks after the injection. Why the excretory organs should fail in this particular is not clear, unless, indeed, the excretion of foreign protein substances cannot be accomplished without simultaneous permeability to native protein. In lieu of the kidneys, or other emunctory organs, the body must get rid of the foreign protein by other means. These means are supplied by the immune reaction.

When foreign protein is parenterally introduced, it is at once seized upon by the cells of the body. Whether all of the cells exercise this function, we do not know, for the reason that the available methods of study necessarily limit our knowledge. We know that if we introduce the serum of a rabbit immunized against horse serum into a guinea-pig, the latter animal promptly becomes sensitized towards horse serum. It has been established that this result is due to the appropriation of the rabbit serum by the cells of the guinea-pig, since only anchored antibodies enter into the anaphylactic response. The study of the reaction of isolated muscular tissues in such animals by means of the pharmacological methods introduced by Schultz permits us to ascertain with certainty that a variety of structures anchor this foreign protein, notably the musculature of the uterus, of the intestine, of the gall bladder, and of the urinary bladder, all of which belong histologically to the unstriated group. The striated muscle of such passively sensitized animals does not respond to the antigen, but this does not prove that this tissue

has not anchored the antibody; it is quite possible that the union of cellular antibody with circulating antigen does not upset cellular equilibrium, or excite a cellular response, in striated muscle. When this reaction does occur, the conclusion is obvious that cellular antibody must be present, but should it fail, the opposite conclusion is not justified. Thus we can state with certainty that the smooth muscle of the guinea-pig appropriates foreign protein, but as to whether the other cellular structures of the guinea-pig's body also share in this function, we must suspend judgment.

Instances are known to us of foreign protein which the cells of the body do not appropriate out of the circulation. This has been shown to be true especially of certain toxins. Thus green lizards, the marsh turtle, and some other animals, are possessed of a very high degree of immunity against tetanus toxin. It is characteristic of these animals that the toxin remains in their circulation for a long period of time, even months. Thus Metchnikoff states that

A lizard kept at a temperature of 20°C., and injected with an amount of toxin sufficient to kill 500 mice, at the end of two months still retains in its blood such an amount of the poison that 0.1 cc. will cause fatal tetanus in a mouse.

These animals are insusceptible to the effects of the poison for the reason that their cells do not anchor it. They retain it in their blood for an indefinite period simply because their excretory organs are not adapted to eliminate it. But there is still a third, and most important corollary of this inability of the cells to fix the poison, namely, the complete absence of antibodies from their blood at any time whatever subsequent to the injection of the poison. In order to eliminate foreign protein, the cells must first anchor it; antibodies are then produced, and finally it disappears from the body. In the absence of antibodies, it may remain in the body indefinitely. Similar conditions appear to govern the resistance of white mice and rats to diphtheria toxin. Thus we may conclude that antibody formation, or the immune response, is the necessary condition for the

elimination of certain foreign proteins from the body, and that the prime requisite for antibody formation is the anchoring of the foreign protein by the cells.

It has been stated that the smooth muscles of the guinea-pig's body anchor foreign protein, and this fact is of primary importance in the study of immunology, inasmuch as it has made possible all the recent studies concerning the fate of such anchored antigen, and the mode of reaction of the cells thereto. This fact does not, however, entirely exhaust our knowledge of the tissues that anchor foreign protein. It has been shown that in the dog the liver almost certainly exercises the same function, and, correspondingly, becomes the seat of a reactive alteration of a specific immune character. Moreover, it seems highly probable that the skin in many species plays an important part in this process. Arthus showed, more than fifteen years ago, that the skin of immunized rabbits reacts violently to the subcutaneous injection of the specific foreign protein. Now, a similar, but less violent, result follows in human beings, but with certain characteristics which indicate strongly that the cells of the skin appropriate foreign protein, and produce antibodies thereto. The facts which lead to this conclusion are as follows. If a patient receives an injection of therapeutic serum into the subcutaneous tissues of one arm, nothing may happen except that at the end of about a week there occurs a delayed local reaction characterized by redness, swelling and heat. If, after an interval of two or three weeks, a similar injection is made into the opposite arm, there is likely to be only a slight reaction, or none at all, at the second site of injection, while the first "lights up" again, and becomes extremely sore. It is difficult to explain this phenomenon except upon the theory that the cells at the original site of injection have responded by the production of a large amount of antibodies which remain localized, and respond violently to the antigen, absorbed from the second injection, which reaches them by way of the circulation. A similar reaction may be observed in the conjunctiva after a previous injection of tuberculin. Thus, many of the tissues of the body absorb foreign protein and produce antibodies thereto,

but conditions are such that this can be affirmed with certainty only of a limited number of them. Nevertheless, if we include the smooth muscles, the skin, and the liver in this latter category, it will be evident that these tissues comprise not only all the embryonic layers, but structures of considerable variety, both from the morphological and the physiological standpoint.

It is an interesting fact that the cells of the body never completely abstract from the circulation the foreign protein which has been parenterally introduced. If horse serum is injected into a guinea-pig, it is possible to determine its continued presence in the circulation by means of a precipitating serum, such as that of a rabbit immunized against horse serum. With this method, it can be shown that no matter how small a quantity is introduced, up to the limits of detection, a residue always remains in circulation for several days. If large amounts are injected, the cells may remove enormously greater quantities from the circulation than in the previous case, although much more also remains in circulation. Thus there appears to be a sort of competition between the cells and the blood for the foreign protein, resulting in a more or less proportional distribution. Why the cells do not absorb the protein to the limit of their absorptive capacity is, of course, entirely unknown. As antibodies are formed in excess in the cells, the latter almost certainly appropriate more and more of the circulating foreign protein, until eventually all detectable traces of it disappear.

The anchoring, or appropriation, of foreign protein by the cells represents the first step in the process of elimination. The further stages of this process are in part known to us, in great part, however, still obscure. The method which has been employed to study these stages is that form of anaphylactic reaction known as the Schultz-Dale method. Essentially it consists in suspending the uterus of the sensitized guinea-pig in a bath of Locke's fluid, and of tracing its muscular contractions on a revolving drum. When the antigenic protein, even in minute amounts, is introduced into the bath fluid, there is a sharp contractile response of the muscle. Thus the occurrence of this response constitutes positive proof that the muscle cells possess

an antibody for the protein which causes the response. If, now, the guinea-pig has been passively sensitized by virtue of the preceding injection of the serum of a rabbit immunized against a foreign protein, such as horse serum, the occurrence of a uterine response to that protein affords proof that the cells are in possession of the rabbit immune bodies. As long as the muscle continues to respond to horse serum, we may be sure that the rabbit antibodies are still present in the guinea-pig cells. On the other hand, the presence of rabbit protein whether in the form of normal or of immune serum, in the guinea-pig cells regularly stimulates those cells to the production of antibodies thereto. Therefore, after an interval of time sufficient to permit of this response, the guinea-pig which has been passively sensitized by immune rabbit serum will come to possess antibodies against rabbit serum itself, in virtue of which it becomes hypersensitive toward rabbit serum. The uterus of such a guinea-pig will then respond to rabbit serum. By combining the study of the reaction to horse serum and that to guinea-pig serum given by the same uterus, it becomes possible to determine the relationships, as they exist within the cell, between the foreign protein originally introduced, namely rabbit serum, and the antibodies thereto, produced by the guinea-pig. These studies have given rise to the following conception concerning the relationship of antigen and antibody in the cells.

If a small amount of rabbit serum is injected, say the minimal sensitizing dose of a powerful rabbit-versus-horse serum, as for example, 0.1 or 0.05 cc., this protein continues to be demonstrable in the cells for a period of from six to eight days. During this time the uterine muscle responds to horse serum, but at the end of it no reaction is obtainable. If, however, a larger amount is injected, as for example, 3 cc. of the rabbit serum, its presence can be detected by virtue of its reactivity to horse serum for a much longer period, even up to three weeks. Antibodies produced by the guinea-pig against the foreign protein of rabbit's serum begin to be manifest after about eight to ten days. For a considerable time, therefore, amounting to as much as ten days, the uterus will respond both to horse serum, indicating the per-

sistence of rabbit antibodies, and to rabbit serum, indicating the presence of antibodies to the latter. In other words, antigen and antibodies coexist within the cells during this prolonged period.

The coexistence of antigen and antibody in the cells is not to be taken as implying a condition of stable equilibrium between these factors. As each day goes by, the antigen diminishes in amount and in effectiveness, and the reaction to horse serum becomes correspondingly less marked. Coincidentally, the reaction to rabbit serum becomes more and more pronounced, and eventually the former disappears as the latter reaches its climax. There is no way to interpret these relationships except as indicating the gradual destruction of the foreign protein within the cells by the antibody of those cells. We have here an intracellular process which is comparable with the solution of alien red blood cells by hemolytic serum, as a mechanism for the complete destruction of foreign protein. In the former case, however, the action is rapid, almost explosive, whereas in the latter it is gradual and prolonged.

Aside from the fundamental fact that foreign protein is destroyed by the cells, almost all of the details of this important process still remain to be discovered. We know that the foreign protein suffers functional deterioration previous to its complete disappearance. The reactivity of the anchored immune bodies becomes changed qualitatively, becomes sluggish, in an early stage of the struggle. In this respect they are affected just as diphtheria toxin is affected by antitoxin; when the latter is present in amounts insufficient completely to neutralize the former, the effects of the mixture are not those of reduced amounts of toxin, but of a completely altered, or attenuated toxin. It seems likely that this early attenuation of the functional activity of the anchored foreign protein might well subserve a useful and conservative purpose, if the functional activity were of a kind likely to harm the cell, as in the case of diphtheria toxin.

With the earliest appearance of reactive antibody in the cells, the same material begins to be discernible in the serum, and so, coincident with the intracellular destruction of antigen, there

goes an extracellular precipitation of the circulating antigen in the blood. It is almost certain that this circulating antibody is derived from the reacting cells. Thus, gradually, the response of the cells serves to eliminate all the foreign protein from the body, both that which the cells have taken up and that remaining in circulation. But it is essential to keep in mind the fact that it is the anchoring of the protein which stimulates the cells to react, and which therefore constitutes the first step in the elimination. Where the cells fail to anchor the foreign protein, as in the instances previously cited of the turtle injected with tetanus toxin, no antitoxin is produced, and for an indefinitely long period the foreign protein remains within the body.

In one respect in particular, the cellular response is of great physiological importance. With it goes a marked interference with normal cellular activities, which in human beings takes the form known as serum sickness. There are fever, cutaneous eruptions, joint manifestations, albuminuria, and other untoward effects. In animals, death may ensue. These phenomena indicate that the symptoms of the infectious diseases may depend in no small measure upon the phenomenon which we have just had under consideration, namely the gradual destruction of the foreign protein of the disease by the cellular antibodies. At all events, it must be admitted that the symptomatology of serum sickness, caused by an absolutely non-toxic and innocuous foreign protein, such as horse serum, can in many respects not be differentiated from that of an acute infectious disease. This remarkable fact goes far to bring home the great importance of such processes as are involved in the reaction of the cell to foreign protein, and is essential to the comprehension of some of the most common phenomena of clinical medicine, as well as of cellular physiology in a general sense.





## CUTANEOUS ANTIBODIES IN THE GUINEA-PIG

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In explaining the mechanism of cutaneous reactions it is frequently assumed that the response called forth in the cutaneous tissue by the introduction of an antigen, to which the individual is sensitized, is due to the presence in the skin of antibodies for that antigen. As guinea-pigs, whether immunized or sensitized, do not give pronounced cutaneous reactions, and as, at the same time, these animals are particularly susceptible to anaphylactic shock when an intravenous injection is made into sensitized individuals, it was considered of interest to discover whether or not antibodies can be demonstrated in their skin.

A possible method of establishing the presence of antibodies is found in the precipitin procedure. In employing this test in work with cutaneous tissue it is essential that circulating antibody be excluded. If the blood serum is eliminated from the skin and a positive reaction is then obtained by using skin emulsions as precipitin with an homologous antigen, we have fairly complete evidence that the skin does possess antibodies. Working upon this hypothesis, experimentation was first made upon immunized animals, as it is generally assumed that antibodies are present in the greatest concentration in the tissues of immunized animals.

### EXPERIMENT I

Two guinea-pigs, 55 and 56, were given five intraperitoneal injections of 0.2 cc. of normal horse serum at five-day intervals. Four days after the last injection the guinea-pigs were killed by bleeding. To eliminate the possibility of obtaining a positive

reaction through the presence of circulating antibody, the animals were perfused according to the method of Dale, that is, the pig was cut completely across at the level of the kidneys, the digestive system removed, a cannula inserted into the aorta and 2500 cc. of warm salt solution perfused through the lower part of the animal. At the end of this procedure when the tissues appeared perfectly white, a portion of the skin from which the hair had been removed was excised, weighed, and ground in sufficient saline solution to make a 10 per cent emulsion of skin. A similar emulsion was prepared from a portion of skin taken from a part of the animal that was not perfused. These emulsions were filtered until clear. They were then used with

TABLE 1

		FIG 55	FIG 56
Antigen (horse serum) in.....	Circulation	1: 40	1: 40
	Skin	1: 40	1: 80
	Perfused skin	1: 20	None
Guinea-pig serum in.....	Skin	1: 80	1: 160
	Perfused skin	1: 40	1: 40
Antibody (for horse serum) in.....	Circulation	1: 400	1: 800
	Skin	1: 320	1: 640
	Perfused skin	1: 80	1: 160

a rabbit anti-horse serum in the precipitin procedure for the detection of residual antigen. Precipitin tests were also made, using the tissue emulsions as precipitinogen and rabbit anti-guinea-pig serum as precipitin, to demonstrate whether or not the guinea-pig serum had been entirely removed from the tissues. Titrations for the antibody content of the guinea-pig sera and tissue emulsions were made using normal horse serum as antigen. The results were as indicated in table 1.

Although we had demonstrated antibodies in the skin of immunized guinea-pigs, we were not able by means of the perfusion method of Dale to exclude positive reactions with the tissue emulsions and rabbit anti-guinea-pig serum. It seemed possible that these reactions might be due to an intracellular substance

which could not be removed by perfusion. To decide the question, that is, to demonstrate whether the substance responsible for the positive reaction was due to circulating guinea-pig serum or to an intracellular substance, other immunized guinea-pigs were given an intravenous injection of an heterologous serum, goat serum, and after a short interval were killed by bleeding. If, after perfusion, the tissues of these pigs contained a substance reacting with rabbit anti-guinea-pig serum, but showed no trace of goat serum, it would appear evident that the reacting body was of cellular origin and that it occurred independently of the presence of circulating blood. The following experiment illustrates the procedure employed.

#### EXPERIMENT II

Guinea-pig 78 received five intraperitoneal injections of 0.5 cc. normal horse serum at four-day intervals. Seven days after the last injection the pig received an intravenous injection of 4 cc. goat serum. This pig died almost immediately after the injection; therefore it was impossible to remove the greater part of the blood by exsanguination. Perfusion was performed 3000 cc. of salt solution being used. Sections of perfused and non-perfused skin were excised and emulsions made and filtered. The blood serum and the two skin emulsions were tested by the precipitin method for residual horse serum, goat serum, and for antibody to horse serum. The skin emulsions were also tested for content in guinea-pig serum. The rabbit serum used for detection of goat serum, in common with the other anti-sera, had a titer of 1:25,600.

The results are given in the accompanying table.

It is evident that this experiment supports the results obtained with pigs 55 and 56, in that antibody was demonstrated in the cutaneous tissue. A positive reaction was also obtained with the perfused tissue and rabbit anti-guinea-pig serum. However, while goat serum was demonstrable in the guinea-pig blood and in the non-perfused tissue, it was not present in as low a dilution as 1:20 in the perfused skin. This is indirect evidence that the substance responsible for the positive reaction in the perfused

tissue when titrated with rabbit anti-guinea-pig serum is not due to circulating guinea-pig serum, since the heterologous goat serum was entirely absent. That the goat serum was completely distributed throughout the pig is evident by the high titer obtained for it in the non-perfused skin.

An experiment similar to that with pig 78 was carried out upon guinea-pig 59, immunized to typhoid. In this case, typhoid agglutinins were used as an indicator of antibody content to typhoid and are analogous to precipitins to horse serum in the previous experiments. In other respects the procedure was the same as with pig 78 and comparable results as indicated in table 3 were obtained.

TABLE 2

		PIG 78
Antigen (horse serum) in.....	Circulation	1: 20
	Skin	1: 40
	Perfused skin	1: 20
Heterologous antigen (goat serum) in.....	Circulation	1: 25,600
	Skin	1: 5120
	Perfused skin	None
Guinea-pig serum in.....	Skin	1: 640
	Perfused skin	1: 80
Antibody (to horse serum) in.....	Circulation	1: 800
	Skin	1: 640
	Perfused skin	1: 320

Having demonstrated the presence of cutaneous antibodies in immunized pigs, the same procedure was employed with a sensitized animal. Pig 79 was sensitized upon March 3 with 1/200 cc. of horse serum. On March 28, twenty-five days later, the pig received 4 cc. of goat serum intravenously, and five minutes later was killed by bleeding. The results of this test will also be found in table 3. They indicate that while there is no antibody to horse serum in the circulation, it is present in slight amount in the cutaneous tissue.

In reference to the point made in the early part of the work that the skin of guinea-pigs perfused according to the method used by Dale does contain a substance which reacts with rabbit anti-guinea-pig serum in the precipitin procedure, it was considered of interest to titer a uterine muscle perfused for the demonstration of anaphylactic antibody for its content in guinea-pig serum and to compare the result with a similar titration made upon perfused skin. As it has been claimed that the response of the uterine muscle of a sensitized guinea-pig to the specific antigen is due to cellular anaphylactic antibody, we considered

TABLE 3

		FIG 59, TYPHOID IMMUNE	FIG 79, SENSITIZED TO HORSE SERUM
Residual antigen in.....	Circulation	.	None
	Skin		None
	Perfused skin		None
Heterologous antigen in.....	Circulation	1: 12,800	1: 12,800
	Skin	1: 320	1: 640
	Perfused skin	None	None
Guinea-pig serum in.....	Skin	1: 320	1: 160
	Perfused skin	1: 80	1: 80
Antibody in.....	Circulation	1: 5,120	None
	Skin	1: 1,280	1: 80
	Perfused skin	1: 640	1: 80

that a comparison of results of the uterine reaction and the antibody content of the skin in the same animal would also be of value.

## EXPERIMENT III

Figs 57 and 58 sensitized to horse serum were perfused, every precaution being taken to retain the tonicity of the uterine muscle. The uterus was removed and one horn suspended in Locke's solution. The other horn was emulsified in saline solution. At the same time a piece of perfused skin was excised

and made up to a 10 per cent emulsion with saline. Upon the addition of 0.1 cc. horse serum to the bath of Locke's solution in which the first horn was suspended there occurred in both animals an immediate and prolonged contraction. Both the uterine emulsions and the preparations of perfused skin were tested for content in antibody to horse serum and for the presence of a substance reacting positively with rabbit anti-guinea-pig serum.

TABLE 4

		FIG 57	FIG 58
Anaphylactic response.....		+	+
Precipitins to horse serum in .....	Circulation	None	None
	Perfused uterine muscle	1: 80	1: 80
	Perfused skin	1: 160	1: 80
Guinea-pig serum in.....	Perfused uterine muscle	1: 80	1: 160
	Perfused skin	1: 80	1: 80

It is clear that not only was anaphylactic antibody demonstrated in the uterus, but precipitins were also found in the uterine emulsions and the skin preparations. As Dale's experiment with the uterine muscle has been considered conclusive proof that the anaphylactic reaction is due to cellular antibodies, the fact that the skin emulsions, and the perfused uterine preparation as well, did contain bodies reacting positively with rabbit anti-guinea-pig serum does not invalidate our conclusion that the skin of guinea-pigs, both immunized and sensitized, contains antibodies.

## THE ABSORPTION OF ANTIGEN: A REPETITION OF WORK REPORTED BY DOERR AND PICK

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The question of the parenteral absorption of antigen has been studied by Doerr and Pick (1). By comparing the reaction in normal and sensitized animals they hoped to determine whether or not in sensitized animals the anaphylactic poison was produced from the antigen. Doerr and Pick injected rabbits, sensitized and normal, with anti-cholera horse serum intravenously. The rabbits were bled at intervals and the sera tested for precipitable horse serum and for agglutinins for the cholera vibrio. Their titrations for precipitinogen and agglutinins indicate, in their opinion, that there is no essential difference in the rate of disappearance of antigen from the circulation of the normal and sensitized rabbits.

To approach the subject from a somewhat different point of view, Doerr and Pick measured the rate of absorption of antigen from the peritoneal cavity of normal and sensitized guinea-pigs. The amount of antigen absorbed into the circulation was titrated as in the rabbit experiments. Their results indicated that the transfer of antigen from the peritoneum to the circulation proceeded much more readily in the sensitized pigs.

From these facts Doerr and Pick conclude that the rate of antigen absorption in sensitized animals, as compared with normal animals, is such as to preclude the possibility of the formation of an anaphylactic poison through the degradation of antigen in situ.

The conclusions which they derived from their experiments have been contested by Friedberger and Lurà (2), and by Römer

and Viereck (3), but in both cases the criticism rests upon the interpretation of the results rather than upon the results themselves.

The procedure of Römer and Viereck differed from that of Doerr and Pick in very essential respects. They employed guinea-pigs in the place of rabbits for the intravenous injections, and measured the absorption of antigen by the loss in diphtheria antitoxin content. Their results indicated that the power of antigen absorption in sensitized pigs greatly exceeds that of normal animals.

Recently Hempl (4) has reported work combining the procedures of Doerr and Pick and Römer and Viereck, arriving at results which she interprets as supporting the latter. Inasmuch as her technic does not conform to that of Doerr and Pick her results do not disprove their conclusions.

It is not our purpose to enter into a discussion of the construction placed upon these facts by any of the authors. Our interest lies in the absorption of antigen in normal and sensitized animals. We have repeated the work of Doerr and Pick upon both guinea-pigs and rabbits, conforming to their technic in every particular except that the antigen employed was an anti-dysentery serum with a titer of 1:25,000, a value considerably higher than that of the anti-cholera serum used by them.

In the accompanying table will be seen how closely our results conform to those of Doerr and Pick upon rabbits. The loss in precipitinogen and agglutinins from the circulation is expressed in percentage rather than in actual values.

HOUR	PRECIPITINOGEN IN SENSITIZED RABBITS		PRECIPITINOGEN IN NORMAL RABBITS		AGGLUTININS IN SENSITIZED RABBITS		AGGLUTININS IN NORMAL RABBITS	
	Doerr and Pick	Our results	Doerr and Pick	Our results	Doerr and Pick	Our results	Doerr and Pick	Our results
1	42.5	37.5	25.0	25.0	33.7	37.5	25.0	16.0
6	56.2	50.0	47.5	31.0	56.2	50.0	47.5	25.0
12	80.0	75.0	70.0	62.5	75.0	62.5	62.5	56.2
24	87.5	91.6	80.0	75.0	80.0	82.0	70.0	67.5
48	93.7	91.6	87.5	84.2	90.0	96.5	82.5	78.0



In general a considerable degree of similarity is apparent. The chief difference lies in the fact that the degree of absorption obtained by Doerr and Pick for both sensitized and normal rabbits is slightly more pronounced for the earlier periods than that obtained by us. A possible explanation of this difference lies in the fact that the amount of antibody introduced by us was far in excess of that used by Doerr and Pick, although the actual amount of serum employed was the same. Assuming that an animal is capable of absorbing only a definite quantity of antigen within a unit of time it is apparent that the percentage absorption of antibody would vary with the antibody concentration. In this connection the work of Walbum (5) is significant. He states that the protein content of a serum influences the rapidity of absorption under certain conditions. He states that immediately after an intravenous injection the content of the blood in agglutinins is the same, regardless of the concentration of the serum. However, the agglutinins of a concentrated serum disappear from the blood more slowly than those of a serum of normal concentration.

A comparison of our results with those of Doerr and Pick upon guinea-pigs is given herewith. The agglutinin and precipitable horse serum appearing in the circulation are expressed in percentage values.

HOUR	PRECIPITINOGEN IN SENSITIZED GUINEA PIGS		PRECIPITINOGEN IN NORMAL GUINEA-PIGS		AGGLUTININS IN SENSITIZED GUINEA-PIGS		AGGLUTININS IN NORMAL GUINEA-PIGS	
	Doerr and Pick	Our results	Doerr and Pick	Our results	Doerr and Pick	Our results	Doerr and Pick	Our results
1	25	50	0	10	25	50	0	8
2	100	100	50	50	100	75	25	19
6	75	100	50	75	75	100	50	56

Our results correspond fairly closely with those of Doerr and Pick. This is to be expected in spite of any possible differences in concentration of the sera used, since, according to Walbum, the absorption of agglutinin from the peritoneal cavity seems to be independent of the protein concentration of the injected serum. The only essential differences in value are found in the results

of the first hour. During this period the absorption of serum in our work was greater than that of Doerr and Pick. Nevertheless, the significant point is that the relationship obtaining between the values for the sensitized and normal animals was very similar in our work to that of Doerr and Pick.

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# AN ANTIGENIC CLASSIFICATION OF THE GROUP IV PNEUMOCOCCI<sup>1</sup>

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A biologic classification of pneumococci into four groups has been established by the work of Dochez, Gillespie, Cole and Avery. A study of type IV, which has been considered a heterogeneous series of independent strains, was undertaken at The Presbyterian Hospital in connection with an investigation of post-operative pneumonia, in which the type IV pneumococci have been found to be an important etiological factor. Many of the strains examined have been obtained, by mouse passage from sputum or saliva of surgical cases before operation and may be considered normal mouth inhabitants. The others have been recovered from the sputum of post-operative pneumonia cases, of pneumonia cases in the medical wards of the hospital, of bronchitis cases, from lung cultures at autopsies, from blood cultures, from spinal fluid, from chest fluid and from abscess cultures. All the strains when isolated failed to react with serum of types I and II. Agglutination, the method that has given such satisfactory results in the hands of the above mentioned workers, has been used for differentiating the strains. Immune serum has been obtained by successive inoculations of rabbits. Only sera agglutinating their homologous strains through at least a 1:80 dilution have been used, most of the sera agglutinating through 1:160. The tests have been performed with equal parts of serum and culture. Positive reactions have been confirmed by tests in dilutions of 1:2 to 1:160. Thirty-seven immune sera have been used and ninety-four cultures have been grouped. Not all cultures have been tested against

<sup>1</sup> Read before American Association of Immunologists, April 6, 1917.

all sera, but enough tests have been performed to demonstrate twelve distinct groups, the members of which cross-agglutinate with each other but not with other groups. Some strains, immune serum for which has not yet been obtained, have not been agglutinated by sera of any of these groups, which probably do not include all type IV pneumococci. Strains cannot be excluded from these groups by lack of agglutination with the sera of these groups, as non-reversible reactions occur. When agglutination does not take place, an immune serum of the unclassified strain should be used against the grouped strains. The largest group thus far found, designated A, contains seventeen strains, seven from the sputum of pneumonia cases and ten from normal mouths. Group B contains thirteen strains; three from the sputum of bronchitis cases, one from the sputum of a post-operative pneumonia case and nine from normal mouths. The strain from the post-operative pneumonia case and one from the pre-operative sputum of a patient who developed post-operative pneumonia were agglutinated by the patient's serum. In group C are twelve strains, two from pneumonia sputum, seven from normal mouths, one from a blood culture, one from the chest fluid of an empyaema case and one from a thyroid abscess. Group D consists of nine strains; two from pneumonia sputum, one from bronchitis sputum, five from normal mouths and one from the sputum of an asthma case whose serum agglutinated the strain. In group E are eight strains; two from pneumonia sputum, one from bronchitis and five from normal mouths. F is a group of eleven strains, all differing somewhat in their cross-agglutination reactions but apparently closely related. Some of these strains are now slightly agglutinated by type II serum, though no reaction was noted when they were first tested, owing possibly to a difference in the strength of the serum. As these strains are more strongly agglutinated by the sera of strains that have shown no relationship with type II than they are by the type II serum, it seems proper to classify them as IV's rather than as atypical II's. Only three F strains were derived from normal mouths; one was from the sputum of a bronchitis case, six were from pneumonia sputum, three agglutinated by the serum of

post-operative pneumonia cases, and one was recovered from the lung culture of a lobular pneumonia case. Group J, cross-agglutinating slightly with F, not at all with II, contains eight strains; one from the spinal fluid of a meningitis case, one from a pre-operative sputum, one from the sputum of an empyaema case, two from bronchitis sputum, and three from post-operative pneumonia sputum, one of which was agglutinated by the patient's serum. Group G consists of six strains; one from pre-operative sputum, and five from pneumonia sputum—one from a post-operative pneumonia case agglutinated by the patient's serum. In this group, as in F, are some strains slightly and irregularly agglutinated by type II serum. The other four groups contain two or three strains each, all from sputum except one that was in the blood culture of a post-operative pneumonia case.

A few absorption experiments and complement fixation tests that have been performed have confirmed the grouping by agglutination. An antigen containing two of the F strains that were agglutinated by II serum gave no complement fixation with II serum, while it did give fixation with sera of group F. This confirms the findings of Avery, who said in volume twenty-two of the *Journal of Experimental Medicine* concerning II X: "Serum type II fails to protect against organisms of this subgroup, and inasmuch as specific protection is regarded as the ultimate criterion for classification, it is doubtful whether organisms of sub group II X are of sufficiently close relationship to be included within group II."

#### CONCLUSIONS

Type IV pneumococci are classifiable.

Cross-agglutination tests with ninety-four pneumococcus IV strains, forty-six from normal mouths and forty-eight from other sources, indicate that at least twelve definite groups exist.

Some members of these groups serve as connecting links between type II and type IV; but, owing to a closer relationship with groups of the latter, they should be classified as pneumococcus IV rather than II X.



## THE VASOMOTOR DEPRESSION IN CANINE ANAPHYLAXIS

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Received for publication, May 18, 1917

In an earlier paper (1) it was shown that the coagulability of the blood in anaphylactic dogs is due directly to a reaction on the part of the cells of the liver. At the same time the suggestion was offered that the vasomotor depression might be due to a local congestive reaction of the liver, leading to a depletion of the general circulation, and in consequence, to a marked fall in blood pressure. This view was based on the observation that in very acute shock in dogs the liver alone of the abdominal organs presents an intense grade of congestion. The following experiments are in support of that suggestion.

A dog is sensitized actively by means of the intravenous injection of horse serum. After an interval of three weeks the animal is etherized and the abdomen is opened by a median epigastric incision. With a fine needle a drop of a 10 per cent solution of horse serum is injected very superficially into the liver. Immediately the site of injection becomes the center of a marked zone of congestive reaction. The injection of egg white produces no such result, although the liver may give a crossed reaction to rabbit serum. Dogs actively sensitized to egg white, however, give a similar hepatic reaction to that antigen, and none to horse serum. Normal dogs present no such reaction to either substance.

The same type of experiment gives a more convincing result when carried out on a somewhat larger scale.

A dog sensitized to horse serum is laparotomized as before. An injection of a small amount of horse serum is then given into

one of the branches of the portal vein, preferably that going to the left median, or left lateral lobe. A striking reaction takes place at once. The injected lobe becomes intensely congested; its color turns to a cyanotic hue, and it becomes turgid with blood. The rest of the liver retains its normal appearance.

These experiments indicate that the congestion of the liver is a purely local phenomenon. Taken in conjunction with the negative results of the transfusion experiments previously reported, they suggest that this is the only mechanism which takes part in the production of the vasomotor shock of anaphylaxis.

#### REFERENCE

- (1) WEIL, R.: *Proc. Soc. Exper. Biol. and Med.*, 1917, 14.



# PROCEEDINGS OF THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS

FOURTH ANNUAL MEETING, HELD AT THE ACADEMY OF  
MEDICINE, NEW YORK CITY

*April 6-7, 1917*

The President, Dr. Richard Weil, in the Chair.

## 1. THE RELATIONS BETWEEN ANTIGEN AND ANTIBODY IN THE LIVING ORGANISM

*Richard Weil:* (President's address, see this volume, page 399.)

## 2. THE TREATMENT OF DERMATITIS VENENATA BY VACCINES

*Albert Strickler:* This study was undertaken with the object of throwing some light on a number of factors relative to dermatitis venenata: (1) the elaboration of an endermic skin test; (2) to determine whether the intramuscular injection of the homologous vegetable toxins would influence the course of dermatitis venenata; (3) whether it is possible to desensitize individuals by the injection of the homologous vegetable toxins and (4) whether complement-fixing bodies could be found in the sera of patients that have had an attack of dermatitis venenata and have been treated by the injection of the homologous vegetable toxins.

The vegetable toxins of ivy, sumac and nettle were isolated and these were employed in our experiments. In all, twelve patients were studied with the following impressions:

1. We have reason to believe that the intramuscular administration of the toxins of the various poisonous plants has an influence on the course of dermatitis venenata.

2. By no means do we advocate this procedure as a routine method of treatment except in extremely severe cases, or where there is a desire to attempt desensitization.

3. From our own observation and the experience of others, we believe that the immunity in dermatitis venenata is a tissue immunity, fleeting in character and one that has to be frequently renewed.

4. It is our impression that it is possible to develop a skin test in dermatitis venenata, and that it is possible to desensitize individuals against plant poisoning.

5. It is our impression that complement fixation is absent in dermatitis venenata.

## DISCUSSION

*A. Parker Hitchens:* These results of Dr. Strickler recall the work of Dr. Ford of Johns Hopkins, seven or eight years ago, who studied the substance in *Rhus toxicodendron* responsible for the dermatitis. This substance in suspension was injected into horses and an anti-*Rhus tox* serum produced. I observed a number of patients upon whose lesions this serum was applied locally; the result was disappearance of the rash over night. This also seemed to show that an immunity reaction followed the injections into the horses just as Dr. Strickler's vaccines apparently cured his patients. I do not think Dr. Strickler is right in using the word "toxin" to describe his extracts.

*A. F. Coca:* I think that Dr. Hitchens is correct in his statement that the active principle in these plants is not a toxin. The injection of these substances into the normal individual does not produce a poisonous effect. The idiosyncrasy of individuals to poison ivy is the same as sensitiveness to pollens, or to any other protein. The resistance of individuals after treatment is not immunity; what takes place cannot be called an immunization process, but it is a desensitization similar to that observed in persons suffering from hay fever. The short period required, as observed by Dr. Strickler, for the disappearance of the symptoms would indicate that the process is a desensitization rather than an establishment of a real immunity by the formation of antibodies. It seems that the application of the antigen through the body fluids is different in its effect on the sensitive cell from the application locally from the outside. I would like to ask Dr. Strickler whether the individuals showed positive skin tests after the treatment. Dr. R. A. Cooke has found that the skin test will still be positive after the successful treatment with the pollens to which they are sensitive, although the ophthalmic test becomes very quickly negative.

*Joseph Head:* I would like to ask Dr. Strickler if the injection of the poison is attended with any soreness.

*Dr. Strickler:* With regard to the last question, I can state that when the test was made with small doses, below 0.4, there was no soreness, but beyond 0.5 there was considerable soreness. A few days ago I gave a student a dosage of 1.2 cc. and she had a very sore arm for four days. I tried to immunize, or desensitize her in order to have another case to report before the meeting.

As regards the skin reaction, I have not done this test on any individual after the treatment. I think it is a very good suggestion and I hope to carry out that idea in the coming series of cases. It can be said in regard to dosage that the soreness increases in proportion to the size of the dose.

### 3. THE EPIDEMIOLOGY OF BACILLARY DYSENTERY

*Wilson G. Smillie:* (Published in full in the American Journal of Diseases of Children, April, 1917, 13, 337.)

## 4. STUDIES ON THE ISOLATION OF STREPTOCOCCI FROM RABBITS

*Edgar T. H. Tsen:* In our investigation of the relation of streptococci to poliomyelitis in which etiological significance was claimed by E. C. Rosenow, we have made cultures of the brains of 23 out of the 54 rabbits which we have studied. Of these 23 rabbits, 5 were inoculated with glycerinated poliomyelitis virus from monkeys, 1 was inoculated with the brain emulsion of a rabbit that died after an inoculation with poliomyelitis virus, 2 were inoculated with streptococci from monkeys, 2 were inoculated with streptococci from rabbits that died after inoculations with poliomyelitis virus, 1 was inoculated with streptococci from a rabbit that died after an inoculation with streptococci, 2 were syphilitic and 9 were normal.

From the brains of all the rabbits that were inoculated with either poliomyelitis virus, streptococci, or *treponema pallida*, we have isolated streptococci and, sometimes, other Gram-positive diplococci. Out of the 9 normal rabbits we have obtained growth of the same kind of microorganisms from the brains of 6.

A part of the tubes inoculated in each case was prepared by methods simulating as exactly as possible those employed by Rosenow.

It was evident from our own findings, as well as those obtained by others, that streptococci can very often be found in many parts of the body in many diseases for which they are not specifically responsible. Moreover they can sometimes be isolated from some tissues of normal individuals. It has been shown by a number of investigators that, even in perfect health, living bacteria may enter the blood and lymph streams; but under these conditions they, or rather most of them, are continuously destroyed after their entrance. We have in our laboratory isolated both anaerobic and aerobic diphtheroids and also small staphylococci from the kidneys of normal rabbits. It thus seems to be proved that the blood and tissues of healthy individuals are not actually, but only potentially, sterile.

The lack of reports on the isolation of pyogenic cocci from normal brains does not prove that they do not at times invade this part of the body. It only means that no attempt has been made to search for them. In diseased conditions where cultures have been made, the presence of these cocci in the brains and cords, spinal fluids and blood has been reported more than once. In 1916 Rosenow reported the findings of streptococci and staphylococci in the nervous system in many nervous diseases other than poliomyelitis. We have found streptococci and small diplococci in the brains of syphilitic rabbits.

Finally our isolation of streptococci cannot be ascribed to air contamination as we have found them in the brains of so many animals. Further, we have found growth more frequently, or, to express ourselves more accurately, growth in about two-thirds and sometimes even in all the tubes inoculated with brains from sick rabbits; while from normal brains we have found growth in only one or, at most, two tubes out of sixteen. Moreover, such cocci have also been found by Flexner and

Noguchi and Rosenow, but they interpreted their findings in different ways. Flexner and Noguchi in their work on poliomyelitis regarded these cocci as contaminations. Rosenow, on the contrary, regarded them as the specific causative agents of many diseases.

Our own interpretation is different from both. We think, in the case of poliomyelitis, these cocci are neither extracorporeal contaminations, nor the specific causative agents of the disease. We regard them as organisms, probably saprophytic in the normal body, which, under conditions of lowered resistance in the course of disease from other causes, acquire the power of more extensively invading the tissues.

#### DISCUSSION

*John A. Kolmer:* I am very glad to hear Dr. Tsen's report, particularly in reference to poliomyelitis. During the recent epidemic of poliomyelitis in Philadelphia, Doctors Freese, Brown and myself found that streptococci and diplococci may be found widely disseminated in the tissues after death; and not only in the brain and cord, but also in the internal organs as well. While these organisms may have a low degree of virulence, they have not produced poliomyelitis in experimental animals. It is possible that they possess a certain degree of virulence and that in poliomyelitis they may occur symbiotically with the true micro-parasite and they may play a certain rôle in the pathogenesis of poliomyelitis as streptococci do in scarlet fever.

*Hans Zinsser:* I would like to support Dr. Tsen's remarks and to say that I don't think he intended to convey the impression that he feels that these organisms are purely saprophytic, but simply that they are not the etiological factor of the disease. But because of the existing disease they are probably able more readily to invade the body extensively than they could do in healthy animals. Whether they are contributory causes to the symptoms of the disease it would be pretty hard to say as yet. I am not prepared to make any statement at all in regard to this point.

#### 5. SOME OF THE CONCLUSIONS DRAWN FROM A COMPARATIVE STUDY OF DIFFERENT METHODS OF PERFORMING THE WASSERMANN REACTION

*J. Bronfenbrenner and M. J. Schlesinger:* That the Wassermann reaction is not specific has long been known and there are no absolute measures that can be used to standardize it, but the results of the tests should not show any more variations than those due to variations of diagnosis. The responsibility for discrepancies in reports from different laboratories is divided between the clinician and the serologist. The clinician sending his specimens to the laboratories does not take proper precautions to assure uniformity of conditions to which the serum is exposed before the test is performed, and he does not realize the importance of supplying an adequate history of the case. There

are, however, a large number of incompetent serologists performing the Wassermann reaction whose results cannot be relied on; no serologist can lay any claim to reliability unless he can properly control the performance of his test, which control begins with the proper preparation and titrating of his own reagents. There is a wide diversity of opinion among serologists as to what method should be used in performing the test, and the various methods differ in delicacy.

The serum when possible must be fresh and not inactivated. We presume that the antigen is free from protein and thus the proteotropic power of fresh serum can be disregarded. In cases where crude alcoholic or watery extracts are employed as antigen, the use of fresh serum may lead to non-specific proteotropic reactions.

The procedure we recommend makes use of fresh unheated serum. The complement of the serum is titrated and utilized in the test, guinea-pig complement being added when necessary. We use reprecipitated acetone insoluble lipoids of which one-tenth the anticomplementary dose still contains 10 to 100 antigenic units. The antihuman hemolytic system is used and the erythrocytes are sensitized before being added to detect the presence of free complement. Incubation for fixation of complement is best performed for thirty minutes at 37°C. in the water bath. However, one can use ice-box incubation for ten to fifteen hours as a presumptive test to eliminate the negative sera, and 37°C. incubation for five minutes to eliminate the strongly positive sera. For diagnosis, however, the one-half hour at 37°C. incubation is the best.

#### DISCUSSION

*Hans Zinsser:* I hesitate to discuss this paper because there are many people here who are doing more Wassermans than I am doing, but it seems to me that there are many points in Dr. Bronfenbrenner's paper which are well worth discussion. I quite agree with the Doctor's remark that fresh serum is absolutely necessary, but there are other points which I am not in agreement with. I don't think it a good plan to do the reaction with unheated serum.

*John A. Kolmer:* I agree entirely with Dr. Bronfenbrenner to the extent that crude alcoholic extracts of tissues, with or without the addition of cholesterol, should not be used in conducting the syphilis reaction with active serum, as in Noguchi's test and that of Hecht-Gradwohl. In these reactions a suitable extract of acetone insoluble lipoids serves best as antigen and is least likely to yield non-specific reactions.

*Richard Weil:* In an association of immunologists the advisability of determination of results by clinical data should be called into question. It is a fallacy for the serologists to assume the clinical history as a guide. It does not seem to me that the serologist is entitled to regard the clinical history as a proof of the presence of the disease. These two things should be kept strictly apart. If the serologist finds

the reaction positive, he should so report it, or vice versa, without reference to the clinical findings. I am not regularly engaged in doing Wassermanns, but make this statement simply on general immunological grounds.

*J. Bronfenbrenner:* The essential thing in the process of preparation of the antigen of Noguchi, is that the cholesterol is removed from it, and the more thoroughly this removal is accomplished, the less anti-complementary is the sample of antigen.

To answer Dr. Weil, I wish to repeat that the *only* way for a serologist to standardize his reagents is to do so with a known syphilitic serum on hand. I wish to make it clear again that if a full value of a serological investigation is desired, a good and complete history should be given. Not in all cases is it essential, but there is no doubt the serologist can say a great deal more about his findings in the cases of recent infection, or in cases under treatment, etc., if he knows the history. The reporting of the degree of fixation to a clinician is of little value, as usually he knows less about serology than is necessary to correlate the findings with the history. On the other hand, the serologist should not attempt to make a diagnosis; his duty is merely to report the presence or absence in the serum of the property to give the lipotropic fixation. If the serologist could feel free from the necessity of making a test-tube diagnosis, there would be more reports of doubtful reactions than there are at present, and some of these doubtful reactions could again be better understood if a proper history was available.

#### 6. COMPLEMENT FIXATION STUDIES ON THE GROUP OF ACID FAST BACTERIA

*Jean V. Cooke:* Cross fixation tests, with varying amounts of immune rabbit sera and a number of both pathogenic and non-pathogenic acid fast bacteria as antigens, gave the following results:

1. Immune sera which will give fixation in amounts varying from 0.004 to 0.0002 cc. can be obtained with any of the acid fast bacteria.

2. There is a group fixation reaction in which all members of the group participate when 0.1 cc. of immune serum is used. With sera of very high titer, all organisms will react with 0.01 cc. of serum. As smaller amounts of serum are employed, various members of the group fail to give fixation until near the titer limit there is usually a small group which still reacts. These groups are not always sharply defined.

3. The immune substances are still present and can be detected in serums for more than three years.

#### 7. COMPLEMENT FIXATION WITH THE STREPTOCOCCUS

*A. Parker Hitchens and C. P. Brown:* (No abstract received.—Ed.)

## 8. AN ANTIGENIC CLASSIFICATION OF THE GROUP IV PNEUMOCOCCI

*Miriam Olmstead:* (To be published in full in this Journal.)

## DISCUSSION

*Rufus Cole:* I am naturally very much interested in Miss Olmstead's work on the differentiation of organisms of this group. I would like to ask whether she has tried dilutions of these sera, and also if she finds that the organisms are typical. The study of these types of organisms is going to be very interesting work from an immunological standpoint. It will be interesting to discover whether persons closely associated with each other carry organisms of these non-pathological groups. It remains to be shown whether these atypical kinds tend to become more fixed and whether their occurrence ever produces epidemics. In South Africa the type that causes the disease most frequently there, is not the kind that we usually find.

*Richard Weil:* I would like to know whether the absorption tests followed an absolute parallel with the agglutination tests. Do the different known agglutinating strains show any capacity for absorption whatever?

*Miriam Olmstead:* The strains within any group agglutinated each other 1:80, or 1:160. In some groups there were sub-groups, the type sera agglutinating all strains in the same dilutions, but the sera of the sub-group not agglutinating the type strains. The strains agglutinated by both II and IV sera were agglutinated very slightly by II, 1:2, or 1:10 only. In many instances, no agglutination was noticeable until the tests had stood in the ice box over night. One of the strains that I considered a Group IV organism was also tested at the Rockefeller Institute and placed in Group IV, while a strain agglutinated slightly by II serum and strongly by the IV serum, was called a II-x strain. I might say that few absorption tests have been done, but those few paralleled the agglutination tests.

## 9. FURTHER OBSERVATIONS ON LOCAL RESISTANCE TO SYPHILIS IN REINOCULATED RABBITS

*H. Zinsser, J. G. Hopkins and M. McBurney:* A report on the local resistance to syphilis in a series of reinoculated rabbits was included in a paper published by Dr. Zinsser, Dr. Hopkins and myself in the Journal of Experimental Medicine for November, 1916. I wish briefly to summarize the observations made at this time and to report eight more reinoculations.

In our first series of 17 reinoculated rabbits, although in some cases doubtful nodules in the testicles could at times be felt, in only three instances could spirochetes be demonstrated by puncture. Only one rabbit developed frank lesions containing active spirochetes and in this case the reinoculation was performed less than two weeks after

the apparent healing of the original lesion. In the other rabbits the interval varies from three weeks to a year. These rabbits were inoculated and reinoculated in groups of three or four, controls were always made by inoculating normal rabbits at the same time that the recovered rabbits were reinoculated and with the same material, and these controls were always positive as proved by puncture. Moreover, numerous other normal rabbits were being inoculated with similar material during the course of these experiments, and these developed lesions in nearly 100 per cent of the cases. In the few cases of successful reinoculation, the second lesion was usually different in type and location from the first; that is, a diffuse lesion was succeeded by nodule at one pole of the testis or by chancre confined to the scrotal tissue. The same observation has been made by Uhlenhuth and Mulzer, though differently interpreted by them. It appeared to us, therefore, that while as a rule a previous lesion in a rabbit confers resistance to reinoculation, the successful reinoculation could be explained on the hypothesis that the resistance was local and limited to the tissue previously involved by the disease.

It was with the intention of further testing this hypothesis that a number of rabbits were inoculated in only one testicle, the other normal testicle to be used as a control at the time of reinoculation. It was found, however, that frequently both testicles showed lesions although only one had been inoculated, or that the only demonstrable lesion occurred in the uninoculated testicle. This is in accord with the results obtained by intravenous inoculation, when if any lesions occur they are usually testicular.

Two of the rabbits in the series I wish to report were inoculated in one testicle and reinoculated in both, neither testicle developing a lesion after reinoculation; but in view of the fact that in the case of one of these rabbits the lesion after the first injection developed in the uninoculated testicle, it seems reasonable to suppose that although a demonstrable lesion apparently occurred on only one side, local resistance to reinfection may have developed in both.

Normal rabbits were inoculated for controls, either from the same lesion used for the reinoculation of the recovered rabbits, or from a lesion of the other testicle of the same rabbit, the object of the latter procedure being to inject the recovered rabbits with as large and concentrated a dose of spirochetes as possible. Only lesions showing numerous active spirochetes were used for inoculation and frequent examinations of the rabbits were made after the inoculations. In several cases when the testes felt slightly abnormal, punctures were made and the material was obtained examined for spirochetes.



## DISCUSSION

*Richard Weil:* I would like to ask a number of questions in regard to this work. It seems to be a remarkable phenomenon if it indicates a localized specific immunity. I do not at all question the occurrence of this phenomenon; it goes with what we know of localized immunity in variola, for example. Again, in making repeated serum injections, one sometimes finds that the sites which had received the earlier injection are the ones which light up when subsequent injections are made at a distance, and this seems to me to show that there is excess of antibody at the site of the original infection.

*Hans Zinsser:* In answer to Dr. Weil's remark that this is a remarkable phenomenon, I would like to say that when we first ran across this it was the first evidence we had of traceable immunity in rabbits. You can reinoculate rabbits on the opposite side and get a positive result. We were unable to demonstrate any antibodies against spirochetes in the serum of syphilitic animals or human beings. Culture spirochetes are antigenically quite different organisms and have nothing in common with virulent spirochetes which seem to be entirely unsusceptible to serum effects. This development of a purely localized immunity was the only evidence of change in resistance in reinoculated animals. So far as culture spirochetes are concerned, syphilitic serum does agglutinate culture spirochetes slightly, but we have studied this phenomenon very extensively and are sure that there is not enough difference between normal and syphilitic serum in this respect to warrant diagnostic use of the reaction. This absence of circulating antibodies in syphilitic individuals renders our discovery of a purely localized resistance all the more interesting.

The conception itself is not absolutely new. Kraus' work with variola was similar to this idea of localized resistance to syphilis. He found that the skin as a whole may become immune. When we speak of this localized resistance, we use the word resistance in the sense of indicating no visible lesion. Neither Dr. Hopkins nor Dr. McBurney nor I myself claim that this is necessarily resistance in an active sense. It may be an "allergie," namely, that the tissues that have reacted no longer react to invasion; spirochete may pass through without arousing any reaction—a thought first suggested by Neisser. Perhaps the spirochetes are not actively destroyed by the resistant tissue, but the apparent resistance, i.e., lack of lesion, is merely an indication of inability to react on the part of cells which have once reacted directly with the virus.

#### 10. THE DESENSITIZATION OF GUINEA-PIGS SENSITIZED TO DOG'S SERUM

*Arthur F. Coca:* One of the constant characteristics of the anaphylactic state is the phenomenon of desensitization which may generally be produced in sensitized animals by the intravenous injection of sub-

lethal quantities of the respective antigen, or by the intraperitoneal or subcutaneous injection of multiples of the intravenous lethal doses.

Experimentation along this line has resulted in the following findings:

Guinea-pigs, actively sensitized to dog's serum, could not be desensitized by (1) subcutaneous injections of large amounts (15<sup>1</sup> lethal doses) of the dog's serum; (2) intraperitoneal injections of one-third<sup>2</sup> of a lethal dose; nor (3) the intravenous injection of one-half of a lethal dose<sup>2</sup> of the dog's serum.

However, the desensitization could be accomplished by the slow intravenous injection (over a period of fifteen minutes) of two lethal doses diluted to 2 cc. with physiological salt solution.

#### DISCUSSION

*Hans Zinsser:* Is there any difference between this and the method of Friedberger of slow injection for the avoidance of anaphylactic shock?

*A. F. Coca:* The method I employed for producing the desensitization is, of course, not original and, as Dr. Zinsser says, it was described by Friedberger. I am merely pointing out that it is the only available method of desensitizing guinea-pigs that have been actively sensitized to dog's serum.

#### 11. STUDIES ON REDUCING ACTION BY BACTERIA

*B. Aranowitz:* 1. The reducing enzyme is not secreted by the cell.

2. The action of the enzyme is independent of the life of the cell.

3. It is destroyed after fifteen to thirty minutes at 60°.

4. It is held back by the Berkefeld filter.

5. Chemicals, as saponin and sodium-taurocholate, known to affect the cell membrane by making it more transfusible, do not accelerate secretion of the enzyme.

6. Grinding of the bacteria, freezing and thawing with subsequent extraction and elimination of the bacterial remnants and cells does not aid in isolating the enzyme from the cell body.

7. Autolytic processes and digestion in a 2 per cent trypsin emulsion in 0.05 per cent sodium carbonate solution do not contribute to the release of the enzyme by the cell protoplasm. It is possible, however, that during these processes the reducing property is destroyed.

8. The bacteriolytic action of immune serum and complement and in certain cases of complement alone, proved to be of value in separating the enzyme from the cell body, as after the elimination of the unbacteriolysed cells, the perfectly clear fluid showed the presence of the reducing enzyme.

<sup>1</sup> Multiples of the lethal dose as determined by intravenous injection.

<sup>2</sup> This quantity of the antigen produced severe symptoms of anaphylactic shock from which, however, the animals quickly recovered.

9. That the proteins of the media are not necessary for the reducing activities of bacteria is shown by the existence of reduction in cultures grown on protein free fluid media.

From the facts enumerated, it seems that we are justified in considering an enzyme the principle responsible for the reduction reactions in the cell.

For reasons that are apparent it seems most proper to call the enzyme the "oxidation-reduction enzymes," as during their activity both oxidations and reductions take place. It is also notable that probably these enzymes produce their actions by breaking up the molecule of water, and are in this respect comparable to the metals of the Pt group. However, there is as yet no experimental proof of the latter hypothesis proposed by Bach.

The fact that by the bacteriolytic method it was found possible to obtain the enzyme outside of the bacterial cell, tends to indicate that the enzyme is not combined with the protoplasm of the cell, as the breaking up of the membrane gives up the enzyme to the surrounding fluid.

The oxidation-reduction enzymes are very probably connected with important processes occurring during the life of the cell, namely, with those of the cell metabolism, respiration of pigments, production of urobilin, etc., but above all their activity is most important in the so-called nitrogen cycle of the nature.

It is known that hydrogen peroxide is usually formed during the life of the cell as a product of oxidations taking place. The fact noticed during our work, that catalase, an enzyme-splitting hydrogen peroxide, diffuses outside of the cell, may indicate the probability that those enzymes which are designed to protect the cell from the injurious substances surrounding the cell (in this case, hydrogen peroxide) are excreted by the latter; on the other hand, the enzymes which assist the metabolic changes taking place in the cell (oxidation-reduction enzymes, carbohydrases, etc.) are not excreted, but are firmly held by the cell. Catalase seems here to be analogous with other substances excreted by the cell, namely, toxin, hemolysins, etc.

## 12. THE INTRACUTANEOUS REACTION IN INFECTIOUS DIARRHEAS

*Cleveland Floyd and Horace Bohn:* (This paper will be published in full in this Journal.)

## 13. CELLULAR AND HUMORAL FACTORS IN ANAPHYLAXIS AND IMMUNITY

*W. H. Manwaring, Yoshio Kusama and Arthur R. Meinhard:* Our analyses of the anaphylactic and immune reactions by means of perfusion experiments with isolated rabbit and guinea-pig tissues have shown that the hypersensitive and immune, humoral and cellular factors may coexist in the bodies of anaphylactic and immune animals in the following combinations:

a. Cellular anaphylaxis and approximately normal blood conditions. Illustrated: lungs of four-week anaphylactic guinea-pig.

b. Cellular anaphylaxis and humoral anaphylaxis. Illustrated: lungs of fourteen-day anaphylactic guinea-pig.

c. Cellular anaphylaxis and humoral immunity. A seeming paradox. Illustrated: lungs of immunized guinea-pig.

d. Cellular immunity and humoral anaphylaxis. A seeming paradox. Illustrated: heart of anaphylactic rabbit.

e. Cellular immunity and humoral immunity. Illustrated: heart of immune rabbit.

f. Humoral anaphylaxis and humoral immunity. A seeming paradox. Illustrated: blood of partially immunized rabbit.

g. Cellular anaphylaxis and cellular immunity. A seeming paradox. Illustrated: heart of the anaphylactic rabbit which tested in a blood-free condition, shows an increased resistance to certain thermolabile, primarily toxic elements of foreign serum and an acquired susceptibility to certain thermostable non-toxic elements.

It will probably be necessary to reduce anaphylactic and immune reactions to lower physiological terms than those at present employed, before hoping to harmonize these observations with current serological theories.

#### 14. ABSORPTION OF FOREIGN PROTEIN BY ANAPHYLACTIC TISSUES AND HEPATIC REACTION IN ANAPHYLAXIS

*W. H. Manwaring and Harold E. Crowe:* If the lungs of a sensitized guinea-pig are repeatedly perfused with 1 per cent foreign serum, diluted either in Locke's solution, or in 50 per cent defibrinated normal blood, the lungs are thrown into a typical anaphylactic reaction.

Quantitative titrations of the perfusion fluid by means of specific precipitins show no recognizable change in the concentration of the foreign serum as a result of the perfusions.

The titrations, therefore, furnish no evidence of the absorption of appreciable amounts of foreign protein by the anaphylactic tissues, nor of the destruction of appreciable quantities by blood enzymes.

If 1 per cent goat serum in 50 per cent defibrinated normal blood is repeatedly perfused through the liver of a normal, or of an immunized guinea-pig, a slight reduction in the toxicity of the perfusion fluid is usually observed on subsequent test with the isolated anaphylactic lungs. In no case, however, is the detoxicating action of the normal or immune liver sufficient to render the perfusion fluid non-toxic.

If a similar perfusion is made through the liver of an anaphylactic guinea-pig, the perfusion fluid is usually rendered completely non-toxic for the anaphylactic lungs.

This reduction in toxicity is not accompanied by a quantitative decrease in the amount of goat protein in the perfusion fluid as determined by titrations with specific precipitins.

The detoxicating action of the anaphylactic liver is, therefore, apparently due to the explosive liberation of an anti-anaphylactic hepatic internal secretion. The mechanism reminds one of the explosive liberation of depressor substances from the liver of the anaphylactic dog.

#### 15. THE DEMAND FOR AND REQUIREMENTS OF A STANDARDIZED WASSERMANN REACTION

*John A. Kolmer:* Because the Wassermann reaction lacks biological specificity, employs biological substances of varying properties and presents certain technical difficulties, the complement-fixation test for syphilis has yielded indefinite and varying results and has been the subject of many modifications. A standardized technique, withstanding the test of time and yielding similar results in the experience of various workers in various laboratories, would increase the value of this reaction in the diagnosis of syphilis and as a guide in treatment, and increase the confidence of physicians in the reactions.

A standardized technique must remove those errors and sources of error of which we have knowledge, and approach as nearly as possible to a state of specificity; the technique must yield similar results with the same serum in the hands of different technicians in different laboratories; the technique must be as delicate as is possible within the limits of specificity and as simple as possible to meet these primary requirements.

A research aiming to standardize the Wassermann reaction has been instituted in the McManes Laboratory of Experimental Pathology of the University of Pennsylvania, with financial support from Dr. William MacKinney; we have classified the various methods in use and are endeavoring to build up a standard technique by submitting every step to experimental test and selecting that which demonstrates superiority on the basis of experimental evidence.

#### 16. STUDIES IN THE STANDARDIZATION OF THE WASSERMANN REACTION. I. A STUDY OF CERTAIN FUNDAMENTAL FACTORS IN THE WASSERMANN REACTION

*Claude P. Brown and John A. Kolmer:* Experiments were made on:

1. The influence of 0.85 per cent of sodium chloride solution upon serum hemolysis and complement fixation, prepared from tap water, commercial distilled water, single and triple distilled water, prepared with ordinary table salt and commercially pure sodium chloride.

2. The influence of acid and alkali; new and dirty glassware; pipets prepared in various ways and washed and unwashed corpuscles, upon serum hemolysis and complement-fixation.

3. Various means of preserving corpuscles and complement for hemolytic work and their practical value.

17. STUDIES IN THE STANDARDIZATION OF THE WASSERMANN REACTION. II. A STUDY OF METHODS FOR STANDARDIZING ANTIGEN IN THE WASSERMANN REACTION

*John A. Kolmer and Berta M. Meine:* We emphasize the necessity of titrating and standardizing antigens and have studied different methods with various tissue extracts and the technique of a standard method, employing standard and fixed units of measure.

18. STUDIES IN THE STANDARDIZATION OF THE WASSERMANN REACTION. III. A COMPARATIVE STUDY OF VARIOUS METHODS FOR CONDUCTING THE WASSERMANN REACTION

*John A. Kolmer and Toitsu Matsunanni:* In this paper the authors presented the results of comparative tests with different hemolytic systems; also with various methods of primary incubation; comparative tests with different methods of titration and adjustment of the hemolytic systems. A standard technique was described which appears to fulfill the requirements.

DISCUSSION OF THE PRECEDING FOUR PAPERS

*R. Ottenberg:* Anyone who has been working at all on this subject must be filled with enthusiasm for Dr. Kolmer's work in testing the best way of doing the Wassermann reaction. I have been working on the subject quite independently of Dr. Kolmer and have reached practically identical conclusions on all the points mentioned. There is hardly a single statement that I can disagree with. The way he has chosen seems to me to be the right one. One essential thing is to get rid of all preconceived notions as to the best way of doing things.

As to the questions of the hemolytic system to be used, theoretically it seems as if the human system should be the best, but there are several practical difficulties which interfere with its use. There is difficulty in obtaining a high titer anti-human serum, and I have not been able to use it extensively on this account. I have used the anti-sheep system. In using the excellent Thomas and Ivy method, I have found the same difficulty as Dr. Kolmer has when a dose of normal serum was inserted in the complement titration, namely, that normal sera vary so widely in their anti-complementary effect that it is impossible to strike an average every time.

The idea of standardizing antigen against dried positive serum which can be kept permanently, is a very good one. I have been doing work along the same line, attempting to preserve the serum by freezing. The Wassermann reaction should certainly be standardized as anti-toxin units have been standardized. In order to do this, a certain number of essential materials must be standardized. The first is a standard positive serum so that new positive sera can be standardized to it. If we adopt as a standard any positive serum the minimal posi-

tive reaction amount of which is known, we can then continuously prepare fresh supplies of standard by determining, under exactly the same conditions, the minimal positive reacting dose of any new sera that come to hand. With such a positive serum various techniques, such as those of Hecht and Weinberg, or of Bronfenbrenner, can be tried. In this way, perhaps, the confusion as to which is the best way to do the Wassermann reaction can ultimately be cleared up.

*J. Bronfenbrenner:* I am sorry to have missed the major part of Dr. Kolmer's important papers, but from the remarks of Dr. Ottenberg I conclude that among other things the choice of hemolytic system has been discussed. Dr. Kolmer apparently admits the theoretical advantages of anti-human hemolytic system and Dr. Ottenberg agrees with him on this question. They claim, however, that for some reason a preparation of anti-human amboceptor of sufficient titer is impossible. I wish to make a few suggestions which, I am convinced, will remove all the difficulties.

First of all the human cells must be very scrupulously washed free from any traces of serum. This requirement, which holds also when any amboceptor is prepared, is especially important in the case of anti-human amboceptor, for the precipitins against human serum which may be produced in rabbits simultaneously with amboceptor, will bind complement when human serum is used subsequently in the test, thus obscuring the action of amboceptor.

Secondly, human cells are known to be more toxic to rabbits than the cells of some other animals; it is therefore preferable not to inject too much blood. Even a comparatively small amount of washed human blood (2 to 3 cc. of 50 per cent suspension) injected intravenously, gives rise to a quite powerful amboceptor.

As intravenous injections may cause anaphylaxis, one must desensitize the animals, beginning with the third injection by introducing a certain amount of the same blood suspension intraperitoneally (2 to 3 cc. of 50 per cent one-half hour before each intravenous injection). After five to six injections at four days interval and nine to ten days after the last injection, the animals are bled and the amboceptor usually gives a titer above 1500.

To obviate another difficulty connected with the use of anti-human hemolytic system—namely, the preparation and standardization of red cells for the hemolytic test, we advocate the following procedures: The clot of any fresh sample of blood is broken up, filtered through a filter paper and thoroughly washed with saline. This having been accomplished, a sample of a resulting arbitrary suspension of cells is estimated by means of Sahli hemoglobinometer, and diluted with saline to the desired concentration, taking the reading of 75 by Sahli as corresponding to 100 per cent concentration of blood suspensions.

In regard to quantitative test, I wish to say that it is very useful, but I prefer not to make it depend on the amount of complement fixed. The fixability and hemolytic power of complement do not go parallel in different animals, and the respective titers of each vary individually

in guinea-pigs. I therefore prefer to titrate my complement to the constant amount of amboceptor and, having found the unit of complement, I endeavor to determine the smallest amount of a given serum which will fix it (in the presence of a suitable amount of antigen). Thus the amount of antibody in the serum is expressed in terms of volume of this serum. If, for instance, 0.002 cc. of serum is the smallest amount causing complete fixation (= 1 unit of antibody), such a serum has a titer of  $X = \frac{0.1}{0.002} = 50$  (units in 0.1 cc. of serum).

*Miriam Olmstead:* In using the cold fixation method the ice box temperature is important; 8° or 9° is the best temperature.

*Claude P. Brown:* Referring to the preparation of anti-human hemolytic amboceptor, the method of Noguchi is certainly very good if the technique is followed closely. We have been preparing the amboceptor paper for five or six years with satisfactory results. The paper is saturated with perfectly fresh serum and dried rapidly; its hemolytic value remains practically constant while, of course, this is not true of serum, as serum always loses considerable of its hemolytic value.

The corpuscles used for the animal injections must be thoroughly washed before injecting them into the rabbits. The injections are continued until the rabbit serum gives a high hemolytic value. The animals are then bled to death and the paper prepared immediately. Very little of the paper is required for test and the agglutination of the corpuscles will not then enter as a factor.

*David Kaliski:* As Dr. Kolmer has pointed out, there is an urgent need for a standardized test, but it must be emphasized that greater care should be exercised in the individual laboratories to prevent error due to neglect of technique, recording and reporting tests, etc.

In our laboratory we have evolved and elaborated a method of procedure that gives constant and reliable results, but it is necessary to control with the greatest care and accuracy each step in the tests, in the preliminary controls and in the actual doing of the tests.

With regard to the anti-human system, I can speak from experience since I had the honor of learning the technique of the system from Noguchi at the Rockefeller Institute. It is difficult, as the previous speaker has pointed out, to immunize a rabbit against human cells and obtain a titer higher than 1:400 to 1:800. These low titer amboceptors may be dried on filter paper, standardized, and will keep their titer for a very long time, deteriorating very slowly.

In performing the Wassermann test, it is very essential to take cognizance of the presence of anti-sheep natural hemolysin which, as I have pointed out, is a great source of trouble in the test. This natural hemolysin for sheep's cells is present in more than half the sera examined and unless provisions for its detection are made, many positive cases will be missed. A technique to detect by simple means the presence of the hemolysin was first worked out in our laboratory. Ice box incubation at about 8°C. for two to four hours after a preliminary water bath incubation at 3°C. for a half hour, will enable one to detect



as high a percentage of positive cases as the use of a reinforced antigen without the disadvantages of the latter. There will be no non-specific results. In the use of non-inactivated sera, the acetone insoluble antigen of Noguchi should be given preference over the ordinary alcoholic extract.

*John A. Kolmer:* I should be very glad to try out the antigen with the hemolysin of Dr. Bronfenbrenner. The production of potent amboceptor serum has been a drawback. I wish to try desensitization by peritoneal injection a half-hour before intravenous injections. I think fixation by complement has been over-estimated by Dr. Bronfenbrenner. I know pigs vary in adaptability to fixation, but I think the variation in fixation is weaker in groups.

*J. Bronfenbrenner:* Dr. Kaliski states that in Noguchi's laboratory the titer of amboceptor was usually low (not more than 800). I spent considerable time in that laboratory from 1909 to 1913 and at no time did I see an amboceptor of such inferior quality. I wish to repeat that it is quite easy to obtain an anti-human amboceptor of a titer over 1500 to 2000. I might add to what I have said before, that we usually do not kill our rabbits. We bleed them from the artery by Latapi method, remove 40 to 45 cc. of their blood (immediately after bleeding the animals receive 50 cc. of warm salt solution 37°C. intraperitoneally) and put them aside for four to five months. At the end of this time we give one or two intravenous injections of human cells and obtain usually a very strong amboceptor. We found, however, that about 5 to 7 per cent of animals may be unsuitable for preparation of amboceptor of a high titer. Besides, should it be necessary, the amboceptor can be concentrated. If one uses amboceptor dried on paper, one may reimpregnate each paper twice. This procedure, however, was never found necessary; in fact, in our recent shipment of amboceptor to the Belgian government our trouble was that the piece of paper representing two units of amboceptor was so small that it was difficult to handle it.

#### 19. IMMUNOLOGIC STUDIES IN TYPHUS EXANTHEMATICUS

*Peter K. Olitsky:* (See this volume, page 363.)

#### 20. SEROLOGIC STUDIES ON TYPHUS FEVER CONTACTS

*George Baehr:* The following observations were for the most part made in Volhynia, Russia, during the first three months of 1916.

Twenty individuals who had lived in most intimate contact with typhus fever patients were discovered to have developed specific antibodies for the *B. typhi-exanthematici* in their blood after their exposure, although at no time did they show any clinical manifestations of illness. In some instances, agglutinins were demonstrable in dilutions of serum up to 1:500 and complement-fixation was four-plus. In over two hundred and fifty non-typhus controls, who with few excep-

tions had not been in contact with typhus fever patients, complement fixation was never observed and agglutinins were not demonstrable in a dilution above 1:50.

Of the twenty typhus contacts, three after their exposure had had vague general symptoms indistinguishable from an influenza. All the others had been perfectly well. Shortly after their exposure, the sera of these contacts were found to contain specific antibodies in amounts otherwise only observed by us in individuals who had recently recovered from typhus fever. In one instance, the serum was still negative when the patient first came under observation immediately after his exposure, and the subsequent progressive development of the agglutinin curve could therefore be followed in detail. In two other cases which were under observation for a long time, the antibodies only disappeared from the blood after three or four months.

None of these twenty contacts subsequently developed the disease although they continued to live in the midst of a typhus epidemic for some months. It is of interest in this regard to note that Nicolle, Conor and Conseil, Ricketts and Wilder and Anderson and Goldberger have independently noted that monkeys which have been exposed to the bites of infected typhus lice may subsequently develop an immunity without having had fever or any clinical signs of illness.

The general immunologic principle deducible from these observations is probably generally applicable to other infectious diseases besides typhus fever. That mild abortive forms of the various infectious diseases may be followed by an immunity is generally recognized. The suspicion has even been voiced that people may become immune following an exposure to some of the infectious diseases, even though at the time they have shown no signs of illness, but until the present time there has been no direct scientific evidence that this does occur.

Individuals, like those reported here, who have become immune to typhus fever without actually having had the disease, are a serious danger epidemiologically. They may act as carriers of infected lice and be the agents for their distribution throughout a community.

#### DISCUSSION OF THE PRECEDING TWO PAPERS

*John A. Kolmer:* I have had no personal experience with the organism described by Dr. Olitsky, but I believe immunological evidence, as summarized this morning, indicates the etiological relationship of this organism to typhus fever. I was interested in hearing about the reaction of the guinea-pig. I think that is directly in line with our knowledge that the state of anaphylaxis does not of itself indicate that antibodies are in the serum or that the sensitive animal is protected against disease, although hypersensitiveness to a certain bacterial protein and immunity may co-exist.

I would like to ask the limits of agglutination of the microorganism by normal human serum and, secondly, which of the two reactions, agglutination or complement fixation, is the better for determining

whether the individual may have had typhus fever. What is the practical value of active immunization of human beings with this bacillus? We are being asked this question and I would like to know how to answer it.

*Harry Plotz:* Dr. Kolmer's question can be answered, regarding active immunization with the typhus bacillus, by a quotation of the results of studies carried on abroad. Vaccinations were carried out in Bulgaria, Serbia and Volhynia, Russia. In all, 10,000 people were vaccinated, consisting only of doctors, nurses and orderlies—people coming in direct contact with those exposed to the disease. Of the 10,000 vaccinated, 6 contracted typhus fever of whom 2 died.

In the town of Uskub, Serbia, there were five military hospitals, four Bulgarian and one German. The four Bulgarian hospitals were vaccinated while the German hospital was not. Despite the fact that many of the patients of the Bulgarian hospitals were often louse-infected, not one single house infection occurred; while in the German hospital there were thirty-four house infections.

From these observations we are led to believe that although the vaccine does not confer absolute immunity to typhus fever, it is capable of reducing the incidence of the disease.

*John A. Kolmer:* Have you any idea of the duration of that immunity?

*Harry Plotz:* None of our observations lasted over six months.

*Peter K. Olitsky:* Agglutination was sometimes obtained with normal serum in 1/20 dilution and in 1/50 dilution in two cases in which there was no history of typhus fever. Complement fixation is the more valuable means of demonstrating the presence of antibodies in the case of typhus fever. It is more specific.

*George Baehr:* In addition to the fact mentioned by Dr. Plotz that of 10,000 people vaccinated only 6 developed the disease, there were other significant observations made by us relative to the value of prophylactic immunization. In the town of Uskub there were five military hospitals, all of which admitted patients suffering from typhus fever. The staff of four of these hospitals were completely vaccinated, whereas in the fifth hospital none was vaccinated. During the three months of the epidemic no house infections occurred amongst the doctors, nurses or staff of the first four hospitals; in the fifth non-vaccinated institution, however, thirty-four house infections occurred, whereas in the fifth hospital none were vaccinated. during the same period of time. Similar observations were made in other places throughout the Balkans and in Volhynia, Russia, where vaccinations were carried out. Our impression was that the prophylactic vaccination does materially reduce the incidence of the disease.

## 21. THE ABSORPTION OF ANTIGEN: A REPETITION OF WORK REPORTED BY DOERR AND PICK

*G. H. Smith and M. W. Cook:* (See this volume, page 421.)

## 22. THE SPECIFICITY OF INTRACUTANEOUS ABSORPTION

*G. H. Smith and M. W. Cook:* (This paper will be published in full in this Journal.)

## DISCUSSION OF THE PRECEDING TWO PAPERS

*Richard Weil:* The sensitized animals, theoretically at least, are partially immune; and yet, paradoxically, they give evidence of absorbing more alien antibodies than do the normal. Friedberger maintained that the peritoneum was inflamed as the result of the primary treatment, and hence permitted more active absorption. The importance of the question depends upon the relation to serum treatment, and the ability of previously treated individuals to profit thereby. Doerr and Pick's observations would seem to indicate that alien antibodies are not destroyed by sensitized animals, but those of Lewis, of Römer and of Hempl point to the opposite conclusion. I would like to cite my own observations on anti-sensitization. A guinea-pig cannot be sensitized passively by immune rabbit serum, except in enormous doses, if it has previously received an injection of normal rabbit's serum. This observation appears to me to establish the fact that such an animal produces anti-bodies which effectively neutralize subsequently introduced alien antibodies. Moreover this property persists for months. It is conceivable, however, that antibodies cannot be produced against diphtheria, or other antitoxin.

*G. H. Smith:* We have not been so much concerned with the theoretical significance of the work or the proper interpretation as with the validity of the facts presented.

With regard to the apparently conflicting results of Doerr and Pick and Römer and Viereck, it should be noted that direct comparisons cannot be made inasmuch as the technique employed has but little in common.

The only work that can be directly compared is that of Friedberger and Lurà in which, by their own statement, their experimental results confirmed those of Doerr and Pick. Their interpretation is, however, very different.

If the results of Römer and Viereck upon guinea-pigs be compared with those of Doerr and Pick upon the same animal, this can only be done by disregarding the methods of inoculation. Even so, the results of the intracardiac injections of Römer and Viereck and the intraperitoneal injections of Doerr and Pick correspond very closely in the differing rates of absorption in sensitized and normal animals.

If the mode of injection be considered as a constant, i.e., the intravascular route, the guinea-pig experiments of Römer and Viereck, and also Hempl, must be compared with the rabbit experiments of Doerr and Pick. It is true that the results differed greatly, but a comparison of the two is open to objection.

In making such comparisons the actual figures are less significant,

in view of the diverse quantitative relationships employed, than percentage value based upon the quantities introduced.

*Richard Weil:* You used sensitized, not immunized animals?

*G. H. Smith:* Yes, we used sensitized animals.

### 23. CUTANEOUS ANTIBODIES

*M. W. Cook and G. H. Smith:* (See this volume, page 415.)

### 24. CLASSIFICATION OF STRAINS OF MENINGOCOCCI

*G. H. Robinson:* Horses were immunized against type strains of meningococci. Mice were injected with the serum of these horses, and the protective value of the serum against other strains of cocci was determined. By this method, the strains tested fall into two distinct groups, and another heterogeneous group, which as yet has not been completely analyzed.

Of the strains tested, 40 per cent belong in the first and second group and 20 per cent in the third group. Concordant results are obtained by actively immunizing the mice and then estimating the protective value against the strains to be tested.

### 25. THE SELECTION OF HORSES FOR THE PRODUCTION OF DIPHTHERIA ANTITOXIN BY AN INTRAPALPEBRAL TOXIN TEST

*A. Parker Hitchens and E. K. Tingley:* (See this volume, page 395.)

### DISCUSSION

*John A. Kolmer:* I would like to ask Drs. Tingley and Hitchens if they titrated out the serum in negative animals for antitoxin as in the Schick test among persons the negative reaction indicates the presence of natural antitoxin.

*A. Parker Hitchens:* We rather think this does give us an indication of productiveness; 14 per cent of those reacting positively became productive; 74 per cent of those reacting negatively became productive. To those who have never had experience in trying to produce diphtheria antitoxin, I would say that this is the first indication we have been able to get as to whether a horse would produce antitoxin. We have made some observations with regard to natural antitoxin in horses showing negative reactions. One horse was found to have 25 units per cubic centimeter of natural antitoxin. We have not, so far, tested lower than for one-fourth unit per cubic centimeter. No positively reacting horse has shown so much.

*Dr. Collins:* Over what time did this test apply? Did you do the Mallein test; if so, before or after? How long does it take the horses to clear up so that if not acceptable they can be returned?

*A. Parker Hitchens:* We are not depending upon the Mallein test; we think the complement-fixation test is more reliable. The Mallein test is made in the stable from which the horse is purchased, the intra-palpebral tests are made after the horse has been received at Glenolden. We have accepted all horses regardless of reaction so far and may continue to do that to get more data.

*John A. Kolmer:* How long does the reaction last and is there necrosis of the cornea?

*E. K. Tingley:* The reaction lasts about a week and the cornea is not at all affected.

## 26. RELATIONSHIP OF THE SYNDROME OF ANAPHYLAXIS TO THE VEGETATIVE NERVOUS SYSTEM

*F. M. Pottenger:* I shall not attempt to discuss the nature of the anaphylactic antibody, except incidentally, but shall confine my discussion to the medium through which the reaction expresses itself.

Disturbance in visceral function occurs through irritation of the vegetative nervous system, or through chemical substances known as harmones. Normal body reactions are purposeful. The syndrome of toxemia shows as a general stimulation of the sympathetic system, while the syndrome of anaphylaxis shows as a general stimulation of the greater vagus.

The relationship which general anaphylaxis bears to the greater vagus division of the vegetative nervous system suggests a close relationship between it and various clinical phenomena as well as to the phenomena which takes place during visceral inflammation.

# INTRACUTANEOUS REACTION IN INFECTIOUS DIARRHEA

HORACE MITCHELL BAKER

*From the Boston Floating Hospital*

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The object of this research is to give a practical clinical test for the diagnosis of infectious diarrheas in infants and children.

In the study of tissue sensitization and its relation to a bacterial substance, we are confronted with a state of sensitization which is perhaps closely related to anaphylaxis with vegetable proteins, and which has been found to exhibit a considerable degree of specificity.

The pioneer demonstration by Rosenau and Anderson (1) that the anaphylactic reaction could be produced with extracts of bacterial cells, has been repeatedly confirmed, and efforts have been made to determine the degree of specificity. While Delanoe (2) has claimed that there is no specificity whatever in bacterial anaphylaxis, positive results of Holobut and Kraus seem to establish the existence of a marked degree of specificity both with active and passive sensitization. Holobut (3) found that specific reactions could be obtained with *B. typhosus*, *B. coli* and cholera vibrios, and although interaction between colon and typhoid bacillus can be obtained, this is to be looked upon as a group reaction, such as is obtained with agglutinins. Kraus and Amiradzibi (4) also found that passive anaphylaxis was specific and that "anaphylatoxin" could be produced only when the serum of sensitized animals was mixed with the homologous bacterial substances.

In this work bacterial substances are employed to determine the specificity of the dysentery group as a means of diagnosing the infection in its early stages, so that proper treatment can be started at once. In the Boston Floating Hospital the treat-

ment of diarrhea is varied according to the type of infection. If it is due to the dysentery bacillus in any of its form (bacillus of Shiga, Flexner, Strong, or His-Y), a low protein and high carbohydrate diet is given. If the infection is due to the gas bacillus and similar organisms or other organisms such as the streptococcus, the colon bacillus, and *B. pyocyaneus*, a high protein and a relatively low carbohydrate diet is given.

Thus it becomes important that an early differential diagnosis be made, in order that the proper food may be prescribed for the patient.

Even with the special bacteriological technique, adapted to this work, it is seldom possible to make a diagnosis of the organism present within twenty-four hours. In many instances stools are cultured six to eight times, before a diagnosis is made. Thus from one to five days are often required for the bacteriological examination to be completed.

In cases with a typical history of an infection by the dysentery bacillus, in one or all of its forms, it is unusual to isolate the organisms from the stool in more than 85 per cent of the cases; thus the advantage of a clinical test for the differentiation of the diarrheas, caused by the dysentery bacillus, in one or all of its forms, from other infections causing diarrhea, will materially aid in the treatment and will serve as a guide to the bacteriologist. This problem was approached by the employment of a carefully standardized bacterial extract. The organism is grown in large quantities, and suspended in normal salt solution. The bacterial suspension is autolyzed at a temperature of 54° for seventy-two hours. During this process the cellular structure of the organism is more or less completely broken down.

The suspension, immediately following the period of autolization, is quickly evaporated to dryness at a constant temperature of 40°, which thus favors the reduction of the bacterial products to a readily soluble form. This latter step is carried out as follows: the suspension to be evaporated is placed in a flat bottom glass dish over a water bath and an air current is directed over the suspension. The flame under the water bath is protected by a shield to prevent variations in temperature.



By this simple device the temperature is kept constant and quick evaporation to dryness is obtained.

The resultant powder is collected in sterile glass stoppered bottles, which are sealed. For testing purposes the powdered extract is ground up with 66 per cent glycerin in the proportion of 10 mgm. of the powder to 1 cc. of the glycerin.

This glycerin extract of the organisms thus obtained is used as a stock for further dilutions. The proper dilution for testing is determined by careful standardization after the preparation of each specific extract. Standardization is carried out either by the Kjeldahl method, or by the cutaneous scratch, dilutions of 1:20; 1:40; 1:60; 1:100; 1:200; 1:500 being applied.

The test is carried out in a manner similar to the Schick test. The inner side of the fore-arm is cleaned with alcohol and ether. Two minims of the standardized extract (1:100 proved to be the correct dilution with my preparation) are injected intracutaneously at about the middle of the inner side of the fore-arm. The same amount of control consisting of 66 per cent glycerin is similarly injected about 2 inches below the original point of inoculation.

Positive or negative results are shown in six to eighteen hours. The reaction is usually at its maximum intensity in eighteen hours. The typically positive reaction is characterized by an irregularly outlined area of redness from 12 to 15 mm. in diameter and occasionally even 20 mm. in diameter.

In the performance of the test all precautions should be taken to avoid trauma. The tissues of the infant as compared with the adults are so much more sensitive that enough trauma may be caused by careless manipulation to obscure the test. Equal care must be taken to avoid subcutaneous injections. The "Vim" ground glass syringe with a very fine needle has proven the most satisfactory for use in performing this test.

In a small number of cases pseudo-reactions have been observed. These are characterized by a pale erythema with a well defined area that is regular in outline as compared with a positive reaction, which has an irregular outline.

These points should be kept in mind in the application and interpretation of the reaction.

Attempts were made to differentiate the type of the infecting organism as well as its group connection. In the former instance the attempt was unsuccessful in as much as a positive reaction could be obtained, for example, in case of an infection with the Flexner type, by the intracutaneous injection of the extract of the bacillus of Shiga. This demonstrated a close relationship between the two strains and was in harmony with Sonnet's statement "that the agglutinins of the dysentery group could not be differentiated."

Because of this specificity of the group reaction, as well as for the treatment, a positive or negative test gives a clearer understanding as to the method of treatment to follow.

In all cases the cutaneous reaction was checked by the bacteriological findings, the cutaneous reaction being charted, in many instances, several days before the bacteriological report became available. Below is a comparative report of the cases together with the control cases.

*Case 1.* Infant with diarrhea for six days. No blood or pus, much mucus in stools, before entrance. No blood, pus, or mucus after entrance. Bacteriological examination negative. Intracutaneous test negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 2.* Diarrhea for two days. No blood or pus, some mucus in stools, before and after entrance to the hospital. Bacteriological examination, negative. Agglutinins negative. Cutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 3.* No diarrhea. Admitted for regulation of diet. Bacteriological examination negative. Cutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 4.* Diarrhea for twelve days before entrance with blood, pus and mucus in stools before and after entrance to the hospital. Bacteriological examination positive for bacillus of Flexner; cutaneous reaction positive in six hours, to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 5.* Diarrhea for six days. No blood, or pus in stools after entrance. Bacteriological examination negative. Cutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 6.* Diarrhea for eight days before entrance with pus, blood and mucus in stools, continuing for one day after entrance. Bacteriological examination negative for dysentery organisms. Cutaneous test positive in six hours, to the bacillus of Shiga, Flexner, Strong and His-Y. Agglutinins positive for the bacillus of Flexner.

*Case 7.* Diarrhea for three days before entrance. Not known to have had blood, pus or mucus in stools, either before or after admission to the hospital. Bacteriological examination positive. Intracutaneous reaction positive in twelve hours, to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 8.* Diarrhea for seven days before entrance with pus, blood and mucus in stools before entrance. No blood or pus after entrance. Much mucus. Bacteriological examination negative for infectious diarrhea. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 9.* Diarrhea began on day of admission to the hospital, with pus, blood, and mucus in stools, continuing several days after entrance. Bacteriological examination negative for infectious diarrhea organisms. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 10.* Diarrhea for seven days before entrance with a history of blood, pus and mucus in stool which was present after entrance. Bacteriological examination positive for the bacillus of Flexner. Intracutaneous reaction positive in six hours to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 11.* Duration of diarrhea not known. History of blood, pus and mucus in stools before and after entrance. Bacteriological examination negative for infectious diarrhea organisms. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 12.* History of diarrhea for five days, with blood, pus and mucus. No blood or pus after entrance. Bacteriological examination positive for the bacillus of Flexner. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 13.* History of diarrhea for twelve days with blood, pus and mucus in stools. Blood and pus in stools after entrance. Bacteriological examination positive for bacillus of Flexner. Intracutaneous test positive in six hours to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 14.* History of diarrhea for twenty-eight days. No blood or pus, much mucus, before and after entrance. Bacteriological examina-

tion failed to isolate organisms of the infectious diarrhoea group. Intracutaneous reaction markedly positive in six hours to the bacillus of Shiga, Flexner, Strong and His-Y. Positive agglutination for the group.

*Case 15.* History of diarrhea for four days with blood, pus and mucus in stools. Blood pus and mucus for several days after entrance. Bacteriological examination showed the presence of the bacillus of flexner. Intracutaneous test positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 17.* Previous history. No blood, pus or mucus. Bacteriological examination showed bacillus of Flexner present. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 18.* History of diarrhea for seven days with blood, pus and mucus in stools before and after admission. Bacteriological examination bacillus of flexner isolated. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 19.* History of diarrhea for twenty-one days, with pus, blood and mucus. No pus or blood after entrance. Bacteriological examination negative. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 20.* History of diarrhea for two days. Clinically typical no organisms regained. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 21.* History of diarrhea for fourteen days, with blood, pus and mucus in stools, before and after entrance. Bacteriological examination negative. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y. Positive agglutination for the dysentery group.

*Case 24.* Previous history not known. No blood, pus or mucus in stool after entrance. Bacteriological examination showed the presence of the bacillus of Flexner. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 25.* Previous history not known. No blood, pus nor mucus in stools after entrance. Bacteriological examination showed the presence of the bacillus of flexner. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 26.* Previous history of diarrhea for twenty-one days, with blood, pus and mucus in stools. Present after entrance. Bacteriological examination showed the presence of the bacillus of Flexner.

Intracutaneous reaction positive in six hours to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 27.* History of diarrhea for two days with pus and mucus in stools. Blood, pus and mucus after entrance. Bacteriological examination showed the presence of the bacillus of Flexner. Intracutaneous reaction positive in six hours to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 28.* History of diarrhea for seven days with pus, blood and mucus in stools. No blood in stools after entrance. Case clinically typical and treated as infectious diarrhea. Bacteriological examination negative for dysentery organisms. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 29.* History of diarrhea for seven days, before entrance, with blood, pus and mucus in stools, before and after entrance. Case clinically typical of infectious diarrhea. Bacteriological examination negative. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 30.* History of diarrhea for four days before. No blood, pus or mucus before entrance. Much blood, pus and mucus in stools after entrance. Bacteriological examination showed the presence of the bacillus of flexner. Intracutaneous reaction positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 31.* History of diarrhea for five days, before entrance to hospital. No blood, or pus, much mucus in stools before and after admission. Bacteriological report, case typical, organism not isolated. Intracutaneous test positive to the bacillus of Shiga, Flexner, Strong or His-Y.

*Case 32.* History of diarrhea for four days, before entrance with mucus in stools. No blood or pus. Blood, pus and mucus in stools after entrance. Bacteriological examination, no report. Intracutaneous test positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Case 33.* History of diarrhea for seven days with pus, blood and mucus, in stools before and after entrance. Bacteriological examination showed the presence of the bacillus of Flexner. Intracutaneous test positive to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 1.* Entered the hospital with a diagnosis of chronic constipation. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 2.* History of diarrhea with blood and mucus in stools before entrance. Bacteriological examination negative. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 3.* Entered with a diagnosis of malnutrition. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 4.* Entered with a history of loss of weight. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 5.* History of diarrhea for three days, with mucus in stools. No blood or pus, before or after entrance. Bacteriological examination negative. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 6.* Entered with a history of vomiting. No diarrhea. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 7.* Entered with a diagnosis of malnutrition. No diarrhea. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 8.* Entered for regulation of feeding. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 9.* Entered with a diagnosis of malnutrition. No diarrhea. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 10.* Entered for regulation of feeding. No diarrhea. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 11.* History of diarrhea with mucus in stools. No blood or pus in stools before or after admission. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y. Bacteriological examination negative.

*Control case 12.* History of diarrhea for four days before entrance. Much mucus. No blood or pus in stools before or after admission. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

*Control case 13.* Entered with a diagnosis of acidosis. No history of diarrhea. Intracutaneous reaction negative to the bacillus of Shiga, Flexner, Strong and His-Y.

## SUMMARY

In the above series of cases the organism of the infectious diarrhea group was isolated in 55 per cent of the cases. Agglutinins for the dysentery group were found in 12 per cent of the cases which were found to be negative from the bacteriological examination and a characteristic smear, as stained by Gram's method, with a typical history in 25 per cent of the other cases.

The intracutaneous reaction, on the other hand, showed 100 per cent negative results in the control cases. In cases with history of infectious diarrhea positive reactions were obtained in 85 per cent of the cases.

I wish to thank Dr. Henry I. Bowditch, chief of the visiting staff of the Boston Floating Hospital for the free use of the clinic and wards, as well as for the many helpful suggestions which he has given me and also Dr. Cleveland Floyd for his valuable aid and advice.

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# A PLEA FOR THE STANDARDIZATION OF REPORTS OF AGGLUTINATION TESTS

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*From the Agricultural Experiment Station of the Rhode Island State College,  
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Possibly the time has not yet arrived when it is desirable to standardize the methods employed in making the macroscopic agglutination test, although there is an increasing tendency upon the part of bacteriologists to employ the technique presented by Kolmer (1915) in which the dilutions of the serum to be tested start at 1:20 and proceed by doubling to give 1/40, 1/80, 1/160, 1/320, etc.

But in the manner in which tests are reported for publication there is observable a great discrepancy on the part of different authors. For the sake of comparative work it would seem to be of advantage to possess a standardized method of reporting these results, whatever the technique of making the tests may have been.

A review of published results of agglutination tests indicates readily enough that the plus sign (+), for indicating a positive test, and the minus sign (−) or a zero (0), for indicating a negative result, have been commonly adopted. So far as interpreting the signs of a positive or a negative test is concerned, the chief difficulty lies in the use of the minus sign (−) by some investigators to indicate a negative test, and the use of the same sign by other writers to indicate the fact that no test was made. The zero sign (0) has also been employed in both of these senses. This diversity may at times produce confusion in the mind of the reader.

But the chief divergence in the methods of reporting tests lies in the manner of reporting the *degree* of the agglutination

reaction. Here, perhaps, the favorite method has been to employ different quantities of plus signs to show the relative degree of sedimentation or of clearing. Sometimes the maximum grade of reaction (complete sedimentation and clearing) has been expressed by two plus signs, sometimes by three and sometimes by four. It is fortunate for both printer and reader that four seems to be the present limit, since the common method of presentation is to print in the proper column four, three, two (or one) signs, apparently with the view that this scheme is psychologically necessary to enable the reader properly to sense the author's meaning. In the case of other types of reaction, such as the degree of the alkalining of milk, in which the minus sign does not express negation, but a definite and positive chemical or physical change in the medium, the multiplication of minus signs is also sometimes employed, as evinced by a recent publication of the present writer; but even this is unsatisfactory and should be remedied. In either case the use of the method is a hardship for the printer and an abomination to the reader. And in the end the idea involved is no more clearly conveyed than it can be by the employment of numbers, since, in the case of agglutination reaction, the numbers can apply to but one thing—the degrees of a positive test; there are no degrees in a negative test.

So far as detectable and significant grades of a positive reaction are concerned, it seems clear that at least four should be established. There may be, on the one hand, a complete clearing and flocculation or sedimentation of the antigen and on the other hand, provided the range of dilutions of the serum is sufficient to include it, there will be found a tube somewhere in the series, beyond which no significant agglutination can be detected macroscopically. These two tubes may well represent the extremes of the positive test and between these it is usually easy to recognize three intermediate grades. It seems unnecessary at present to multiply the differences beyond this point.

Accepting the use of these grades of flocculation or of clearing, the writer proposes the following scheme for records and for publication of the results.

1. That a complete flocculation and clearing, so that the medium becomes water-clear, be referred to as a complete agglutination, and be expressed by the symbol (C).

2. That the last tube, beyond which no significant agglutination can be observed, be regarded as an agglutination of grade 1, and be expressed by the symbol (1).

3. That between these two extreme grades of reaction there be recorded, if present, three intermediate grades expressed by the symbols (4), (3), (2).

Furthermore, in order to obtain greater unanimity in the expressions of (1) negative reactions and (2) "no test," the writer proposes:

4. That a negative test (i.e., no appreciable agglutination) be expressed by the zero sign (0).

5. That when no test is made in a certain dilution for a certain antigen, this circumstance be expressed by the minus sign or blank (-).

Although usually representing faulty technique in the preparation of the antigen-suspension, it sometimes happens that the control tube shows some degree of sedimentation. This should always be reported in the test, in the column headed "control." There is an unfortunate disregard among investigators in this particular. It frequently happens that an approximate estimation in a certain test can be arrived at only through a knowledge of the degree of sedimentation in the control tube. If the sedimentation is slight the test may still possess significance; while if sedimentation is marked, the value of the test is correspondingly depreciated. In order to improve this situation the writer therefore suggests further:

6. That statements regarding the degrees of sedimentation in the control tube should always be reported in a "control column."

7. That the smallest detectable quantity should be regarded as a *trace* of sediment, and expressed by the symbol (T).

8. That a significant sedimentation (comparable, for instance, with a grade 1 agglutination) should be expressed by the symbol (S).

9. That such a degree of sedimentation as might perhaps invalidate the test should (if the test is reported at all) be expressed by the symbol (SS).

To illustrate more clearly what is meant, the following arrangement of column headings and body is suggested. A fowl typhoid serum is here tested against various antigens.

NO.	ANTIGEN	SERUM (FOWL TYPHOID, NO. 116) DILUTIONS								DATE
	Designation	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Control	
158	<i>B. gallinarum</i> .....	C	C	C	C	C	4	2	0	April 1
40	<i>B. typhosus</i> .....	C	3	2	1	T	T	—	S	April 1
48	<i>B. avisepticus</i> .....	T	0	0	0	0	—	—	0	April 1
17	<i>Bact. pullorum</i> .....	C	C	C	C	3	1	T	T	April 1
9	<i>B. paratyphosus</i> .....	4	3	2	2	1	1	1	SS	April 1
116	Control .....	C	C	C	C	C	C	4	0	April 1

The method of recording as outlined possesses an additional advantage in so far as it affords a simple means of expressing single agglutination tests or the range of such tests when positive. For instance, referring to the tabulation given above, it might be said that the fowl typhoid serum agglutinated *B. gallinarum* (Klein) at C/320, meaning that a dilution of 1:320 of the serum was the highest dilution that afforded a complete agglutination with the named antigen.

Or, it might be stated that the significant range of agglutination of the fowl typhoid serum for the human typhoid antigen was C/20 to 1/160; and for the *Bact. pullorum* antigen, C/160 to 1/640; while the titer of the same serum for its homologous antigen (control test) was C/640.

And again the relative agglutinability of *Bact. pullorum* and of *B. typhosus* antigens in the presence of fowl typhoid serum might be expressed as C/160 : C/20; or the relative agglutinability of *B. avisepticus* and *B. paratyphosus* in the same serum as T/20:4/20.

One circumstance which might diminish the value of such a system of recording results would be the use, as antigens, of bacterial suspensions of varying density. This is not a signifi-

cant obstacle however in view of the possibility of standardizing the antigen-density itself by such methods as have been recommended by MacFarland in his nephelometer, and by others using the barium sulfate method.

To conclude, in the writer's opinion the common employment among investigators of such a system as that outlined above for recording and expressing the results of their agglutination tests would eliminate much confusion in interpretation, and render the results of the tests of different workers comparable to a much greater extent than at the present time.



## STUDIES IN ANAPHYLAXIS

### XX. THE RECIPROCAL RELATIONS OF ANTIGEN AND ANTIBODY WITHIN THE CELL

A CONTRIBUTION TO CELLULAR IMMUNOLOGY

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When foreign protein is introduced into the body it is rapidly appropriated by the cells, which are thus stimulated to the production of antagonistic substances, the so called antibodies. This process eventually results in the destruction of the foreign protein; meanwhile, the interaction of these two factors (antigen and antibody) within the cells gives rise to a serious disturbance of the bodily economy, which constitutes a large element in the symptomatology of the infectious diseases. For these reasons, the details and the mechanism of the cellular process are worthy of careful analysis.

A number of previous papers in this series of studies have been devoted to the experimental demonstration of the fact that antigen and antibody coexist for some time within the cell. In the present paper this contention is fortified by evidence of a character different from that already adduced. Furthermore, an attempt is made to determine directly the influence that these two factors exert upon each other in the cell.

#### I. THE MODIFICATION OF INTRACELLULAR ANTIBODY BY ANTIGEN

##### *Reactions of the partially desensitized cell*

The first problem that was undertaken in this connection concerns the effect of antigen upon cellular antibody. It is a very generally accepted idea that antigen in some manner satu-

rates or neutralizes antibody when the two are brought into contact. According to the terms of Ehrlich's theory, the antigen satisfies the haptophore group of the antibody, and so deprives it of the possibility of further reaction with additional antigen. This explanation would harmonize well with the relations that have been found to obtain for the reactions between precipitin and precipitinogen, which are strictly constant and quantitative (1). Inasmuch as precipitin and sensitizin, or cellular antibody, are identical, it might be assumed that the same type of reaction would be presented by them. On the other hand, toxin and antitoxin do not react in this manner, but, as Bordet and others have shown, in varying and inconstant proportions. In order to determine the type of reaction of cellular antibody with antigen, it becomes necessary to study by quantitative methods the effect of definite amounts of the latter upon the reactivity of the former.

The methods of this study are simple, and are practically predetermined by the character of the problem. Passive sensitization should be employed, in order that the amount of antibody at the disposal of the animal may be accurately controlled; with active sensitization this is not strictly the case. One and the same immune serum must be used throughout, in order that the standard of comparison may not vary. The first step is to determine the minimal amount of immune serum which will suffice to sensitize guinea-pigs passively. One series of guinea-pigs is then sensitized by intraperitoneal injections of this amount. Several other series of pigs are likewise sensitized with various multiples of the minimal sensitizing dose. In each of these series, then, the minimal anaphylactic dose is determined, i.e., the smallest amount of antigen which suffices to produce acute anaphylactic death upon intravenous injection. These data are the necessary preliminaries to the second step of the experiment, which is conducted as follows. Guinea-pigs, sensitized as before, with graded doses, in series, are now given desensitizing injections of various amounts of the antigen, and, after an interval of a day, the minimal anaphylactic doses are again determined for each desensitizing unit. When these data have been experi-



mentally determined, it becomes a simple matter of comparison to discover whether the degree of desensitization is quantitatively proportional to the desensitizing dose. If such is not the case, as, indeed, will be very clearly shown by the results, the mechanism of desensitization must be entirely different from that which governs the neutralization of precipitin by precipitinogen, and the nature of this mechanism must then become the subject of further discussion.

### *Experimental data*

*Sensitizing serum.* The immune serum employed in the following experiments was derived from a rabbit which had been given a series of intravenous injections of horse serum. After a preliminary test had shown this serum to be of high titer, the animal was given a large bleeding, and the serum was kept frozen solid throughout the course of the study, during which it showed no appreciable deterioration. It is worthy of note that such frozen serum should always be completely melted and then thoroughly mixed before being injected into animals, since the titer of partially frozen serum is measurably different from that of the whole fluid.

### *Precipitation titer*

The value of this serum from the standpoint of precipitation and of sensitization may be gauged from the following tables.

TABLE 1  
*Precipitation titer*

	HORSE SERUM					
	1 cc.	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.
S. 265, 0.1 cc. ....	—	++	++++	+++	+	Slight
..... (Prozone)						
S. 265, 0.01 cc. ....	—	—	+	++	+	—
..... (Prozone)						

TABLE 2  
*Minimal sensitizing dose*

GUINEA-PIG	SERUM 265, INTRAPERITONEALLY	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	cc.	
1	0.1	1	†
2	0.05	1	†
3	0.03	1	Moderate symptoms
4	0.03	1	Mild symptoms

In the above series, the guinea-pigs, all of which weighed from 250 to 300 grams, received the intoxicating dose of horse serum on the day succeeding the sensitizing injection.

† = immediate death.

The minimal sensitizing dose, as determined by this series, was 0.05 cc. of serum 265. No amount intermediate between the latter and 0.03 cc. was tested.

*Minimal anaphylactic dose of horse serum after various sensitizing doses*

TABLE 3  
*Sensitizing dose: 0.05 cc. serum 265*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.1	†
2	0.075	†
3	0.05	†
4	0.04	Moderate symptoms
5	0.04	Mild symptoms
6	0.03	Very mild symptoms

In this, as in the three following series, the injections of antigen were given on the fifth day, in order to maintain conditions comparable with those of tables 8 to 17.

TABLE 4  
*Sensitizing dose: 0.075 cc. serum 265*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.035	†
2	0.03	†
3	0.03	Immediate convulsions; recovery
4	0.025	Severe symptoms
5	0.02	Moderate symptoms

 TABLE 5  
*Sensitizing dose: 0.1 cc. serum 265*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.005	†
2	0.003	†
3	0.002	Moderate symptoms
4	0.002	Mild symptoms

 TABLE 6  
*Sensitizing dose: 0.3 cc. serum 265*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.006	†
2	0.005	Immediate convulsion; recovery
3	0.004	Severe symptoms
4	0.003	Mild symptoms

The data included in the foregoing tables are summarized in the following table.

 TABLE 7  
*Summary of 3 to 6*

SENSITIZING DOSE SERUM 265	MINIMAL FATAL DOSE OF HORSE SERUM, INTRAVENOUSLY
cc.	cc.
0.05	0.05
0.075	0.03
0.1	0.003
0.3	0.005

Parenthetically, it may be noted that the table corroborates the conclusions of a preceding study (2), in that the smaller sensitizing doses require larger doses of antigen to kill. This rule, however, appears to be reversed by the final series, wherein a very large sensitizing dose (0.3) was employed; here the killing dose of antigen is found to be somewhat increased as compared with the immediately preceding series. The explanation of this fact is probably to be found in the protective effect of the excess antibody which circulates in the blood. Circulating antibody, as has previously been shown, protects the cellular antibody against the attack of antigen, and naturally raises the minimal anaphylactic dose.

*Minimal anaphylactic dose after partial desensitization*

The second step in the study of the problem consists in determining the minimal anaphylactic dose after partial desensitization of animals passively sensitized by various amounts of serum 265. These experiments may be divided into two groups. In the first group, four different sensitizing doses were employed, whereas the desensitizing dose was not varied. In the second group, the sensitizing dose was the same throughout, but the desensitizing dose was varied. The final injection was given on the fourth or fifth day.

TABLE 8

*Sensitization: 0.05 cc. serum 265. Desensitization on third day: horse serum, 0.01 cc. intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	2	†
2	1	Convulsions; recovery
3	1	Severe, delayed symptoms
4	0.8	Severe symptoms
5	0.6	No symptoms

TABLE 9

*Sensitization: 0.1 cc. serum 265. Desensitization on fourth day: horse serum 0.01 cc. intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	1	†
2	0.7	†
3	0.6	†
4	0.5	Severe symptoms
5	0.4	Mild symptoms

TABLE 10

*Sensitization: 0.2 cc. serum 265. Desensitization on fourth day: horse serum 0.01 cc. intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.6	†
2	0.6	†
3	0.5	Severe symptoms; re-covery
4	0.4	Mild symptoms
5	0.1	No symptoms

TABLE 11

*Sensitization: 0.3 cc. serum 265. Desensitization on fourth day: 0.01 cc. horse serum intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.6	†
2	0.5	†
3	0.4	Mild symptoms
4	0.3	Mild, delayed symptoms

The data obtained in the four preceding experiments may be collated as follows.

TABLE 12  
*Summary of tables 8 to 11*

SENSITIZING DOSE SERUM 265	DESENSITIZING DOSE HORSE SERUM	MINIMAL ANAPHYLACTIC DOSE OF HORSE SERUM
cc.	cc.	cc.
0.05	0.01	1.0 to 2.0
0.1	0.01	0.6
0.2	0.01	0.6
0.3	0.01	0.5

It seems to be characteristic of all four series of tests that the minimal anaphylactic dose, after an intermediate desensitizing injection of antigen, is greatly in excess of the minimal anaphylactic dose as determined in any of the series summarized in table 7. In none of the series shown in table 12 is it less than ten times the largest minimal anaphylactic dose of table 7: to wit, 0.5 cc. as compared with 0.05 cc. Inasmuch as 0.05 cc. was the minimal anaphylactic dose determined after sensitization with the smallest effective sensitizing dose of the immune serum, it is clear that the great increase in the minimal anaphylactic dose seen in table 12 cannot be explained on the theory of neutralization of a portion of the antibody, leaving a residual fraction free to react with the second dose of antigen. Were this the case, an animal sensitized passively by 0.3 cc. of serum 265, and then partially desensitized by the injection of 0.01 cc. of horse serum, would of necessity have a minimal anaphylactic dose lying somewhere between 0.005 cc. and 0.05 cc. of horse serum. The former, 0.005 cc., is the minimal anaphylactic dose after sensitization with 0.3 cc. of serum 265; the latter, 0.05 cc., is the minimal anaphylactic dose after sensitization with the smallest effective dose of serum 265, namely 0.05 cc. Evidently, if part of the 0.3 cc. of serum 265 which has been injected into the guinea-pig is neutralized by an intermediate injection of the antigen, the portion that remains intact, down to a minimum of 0.05 cc., will have the minimal anaphylactic dose corresponding to an equivalent primary sensitizing injection of that amount of serum 265. If, on this theory, 0.27 cc. of the 0.3 cc. is neutralized, the guinea-pig will not respond fatally to any

dose whatever of antigen, since only 0.03 cc. remains free, which is below the minimal sensitizing dose of the serum. If, however, 0.2 cc. of the sensitizing dose is neutralized by the intermediate injection, the guinea-pig will still retain 0.1 cc. of the antibody intact, and the corresponding minimal anaphylactic dose will be 0.003 cc. of horse serum. The facts, however, are entirely at variance with these suppositions. As a matter of fact, a guinea-pig sensitized by 0.3 cc. of serum 265, and then partially desensitized by 0.01 cc. of horse serum, has a minimal anaphylactic dose which is very greatly in excess of that obtained after passive sensitization with any amount of serum 265 less than 0.3 cc. It seems evident, then, that desensitization cannot be explained on the basis of the neutralization or saturation of a fraction of the cellular antibody. *We are, therefore, forced to the conclusion that partially desensitized antibody reacts to antigen in a manner which is quite different from that of pure antibody.*

This conclusion is strengthened by a further study of the data in table 12. It will be seen that the minimal anaphylactic dose after desensitization with 0.01 cc. of horse serum is practically the same, whether the guinea-pigs were sensitized by 0.1 cc., or 0.2 cc., or 0.3 cc. of serum 265. This result again is clearly not in keeping with the belief that there is an accurate quantitative relationship between the amount of antigen employed in desensitization and the amount of antibody thereby neutralized.

The mode of experimentation was further varied by sensitizing guinea-pigs with identical amounts of serum 265, and then desensitizing several series with different quantities of the antigen. The determination of the final minimal anaphylactic dose for each series afforded the basis for instructive comparisons. In the following experiments, the guinea-pigs in all four series were passively sensitized with 0.3 cc. of serum 265. The desensitizing doses were 0.005 cc., 0.01 cc., 0.02 cc., and 0.05 cc. of horse serum respectively. The final tests were always performed on the day following desensitization.

The results in Table 16 are rather irregular. Although one animal which had received 0.4 cc. of horse serum died, two which had received 0.5 cc. recovered, although they manifested

TABLE 13

*Sensitization: 0.3 cc. serum 265 intraperitoneally. Desensitization, third day  
0.005 cc. horse serum intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.4	†
2	0.2	†
3	0.1	†
4	0.05	Moderate symptoms

TABLE 14

*Sensitization: 0.3 cc. serum 265, intraperitoneally. Desensitization, third day:  
0.01 horse serum intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.7	†
2	0.6	†
3	0.5	†
4	0.5	Convulsions; recovery
5	0.4	Moderate symptoms

TABLE 15

*Sensitization: 0.3 cc. serum 265 intraperitoneally. Desensitization, third day:  
0.02 cc. horse serum intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.7	†
2	0.6	†
3	0.5	†
4	0.4	Mild symptoms

TABLE 16

*Sensitization: 0.3 cc. serum 265 intraperitoneally. Desensitization, third day:  
0.05 cc. horse serum intraperitoneally*

GUINEA-PIG	HORSE SERUM, INTRAVENOUSLY	RESULT
	cc.	
1	0.5	Convulsions; recovery
2	0.5	Convulsions; recovery
3	0.4	†
4	0.4	Mild symptoms
5	0.3	Very mild symptoms



extremely severe symptoms. Discrepancies of this sort are not improbably due to an imperfect absorption of the desensitizing dose of the antigen. It seemed advisable to consider the minimal anaphylactic dose of this series as 0.5 cc., which can certainly not be in error to the extent of more than 0.1 cc.

Summarizing the results of the preceding four tables, we obtain the following table.

TABLE 17

*Summary 13 to 16. Sensitization: 0.3 cc. 265 serum intraperitoneally*

GUINEA-PIG SERIES	DESENSITIZING DOSE OF HORSE SERUM	MINIMAL ANAPHYLACTIC DOSE OF HORSE SERUM, INTRAPERI- TONEALLY
	cc.	cc.
1	0.005	0.1
2	0.01	0.5
3	0.02	0.5
4	0.05	0.5

Series 2, 3, and 4 have the same minimal anaphylactic dose in spite of the fact that the desensitizing dose was five times as large in series 4 as in series 2.

Before passing on to the discussion of the preceding data, it is necessary to consider two possible objections to their validity. These objections are based, first, on the complex character of the antigen, and, second, on the complex character of the anaphylactic response as observed in the living animal. It is fortunately possible to repeat the experiment under simpler conditions, comprising, first, the use of a chemically pure antigen, and, secondly, the reaction of the isolated sensitized muscle preparation.

*Results obtained with a simple antigen*  
*Passive sensitization*

The experiments above described were carried out with horse serum, an antigen which has the advantage of being ready at hand and practically universally available. On the other hand, it is open to certain objections, based upon the fact that it is a complex mixture, actually composed of a number of very different

antigenic substances—albumins, globulins, and so forth. On this ground it might be argued that the corresponding antibodies are probably likewise multiple, and that the desensitization experiments are, therefore, too complex to permit of definite conclusions. For this reason the same type of experiment was carried out with a chemically simple antigen, namely crystalline egg albumin.

The crystalline egg albumin was prepared according to the method of Hopkins. The antiserum was derived from a rabbit highly immunized against this antigen by means of repeated intravenous injections. Of this antiserum, 0.05 cc. was the minimal passive sensitizing dose for guinea-pigs weighing approximately 250 grams. The minimal anaphylactic dose of a 5 per cent solution of the crystalline egg albumin solution after such sensitization was 0.004 cc. The results of a series of tests conducted with these two reagents, according to the methods already described, are summarized in the following table. The data are not given in detail, inasmuch as the procedures are identical with those illustrated by tables 1 to 17.

TABLE 18

*Passive sensitization with serum of rabbit immunized to crystalline egg albumin*

SENSITIZING DOSE	MINIMAL ANAPHYLACTIC DOSE	DESENSITIZING DOSE OF EGG ALBUMIN, INTRAPERITONEALLY	
		0.01	0.04
		Minimal anaphylactic dose	
0.05	0.004		
0.2	0.001	0.09	
0.4	0.002	0.08	0.09

It is evident that the results obtained with this simple antigen do not differ in any essential particular from those secured with horse serum. Here again the minimal anaphylactic dose after partial desensitization is very greatly in excess of any minimal anaphylactic dose without desensitization. Again the minimal anaphylactic dose after desensitization with widely differing amounts of antigen shows little variation.

### *Active sensitization*

A further set of experiments was carried out with crystalline egg albumin, in which the animals were actively instead of passively sensitized. This method has certain disadvantages, inasmuch as it lacks the quantitative exactness of passive sensitization. It is based on the assumption that guinea-pigs of equal weight, actively sensitized by identical doses of foreign protein, will react in an approximately similar manner. The data of the experiments will be found to bear out this assumption. Experiments with active sensitization suffer from the further disadvantage that neither minimal sensitization, nor, of course, definite multiples of this degree of sensitization, can be induced. Notwithstanding these drawbacks, it seemed advisable to determine whether actively sensitized animals reacted in a manner comparable to that of passively sensitized animals.

In the experiments in active sensitization, the antigen employed was crystalline egg albumin. Guinea-pigs weighing from 240 to 300 grams received intraperitoneal injections of 0.03 cc. of a 5 per cent solution of this antigen. After an interval of eighteen days the minimal anaphylactic dose was determined by intravenous injection, with the following results.

TABLE 19

*Active sensitization with 0.03 cc. 5% egg albumin. Tested on nineteenth day*

GUINEA-PIG	ANTIGEN, EGG ALBUMIN INTRAVENOUSLY	RESULT
	cc.	
1	0.001	No symptoms
2	0.001	No symptoms
3	0.005	†
4	0.01	Died in two hours
5	0.01	†
6	0.01	†
7	0.02	†
8	0.05	†

This table shows slight discrepancies due to individual variations in the immune mechanism. It appears safe to say that the minimal anaphylactic dose is not greater than 0.01 cc. in the majority of the animals; exceptionally, however, it may be less.

Animals prepared on the same day and in the same manner as those above described were partially desensitized on the eighteenth day by the intraperitoneal injection of the antigen in varying amounts. On the succeeding day the minimal anaphylactic dose was determined. The following table shows the results obtained when the desensitizing dose was 0.01 cc. of the egg albumin solution.

TABLE 20

*Animals sensitized by 0.03 cc. egg albumin, intraperitoneally. Desensitized on eighteenth day by 0.01 cc. egg albumin, intraperitoneally. Tested on nineteenth day intravenously*

GUINEA-PIG	ANTIGEN	RESULT
	cc.	cc.
1	0.05	No symptoms
2	0.05	No symptoms
3	0.1	Mild symptoms
4	0.1	No symptoms
5	0.1	†
6	0.1	†
7	0.2	†
8	0.2	†
9	0.2	†
10	0.3	†

In this series the minimal anaphylactic dose lies between 0.1 cc. and 0.2 cc., and is therefore ten to twenty times as great as in the preceding, undesensitized, series.

Desensitization with 0.005 cc., 0.01 cc., and 0.02 cc. of egg albumin gave practically identical results. The animals were regularly killed by 0.2 cc., and occasionally by 0.1 cc., of egg albumin. After desensitization by only 0.001 cc. of egg albumin, the minimal anaphylactic dose was less than 0.05 cc.; after 0.06 cc. it was greater than 0.3 cc. These data are presented in the following table.

These results confirm the findings with passive sensitization. Not only is the minimal anaphylactic dose greatly increased, but it shows little variation over a considerable range of desensitizing doses of the antigen; in other words, there is no definite proportion between these two values.

TABLE 21

*Sensitization by 0.03 cc. egg albumin, intraperitoneally. Desensitization on eighteenth day, intraperitoneally. Tested on nineteenth day, intravenously*

DESENSITIZING DOSES	MINIMAL ANAPHYLACTIC DOSE OF EGG ALBUMIN
cc.	cc.
0.001	0.05 or less
0.005	0.1 to 0.2
0.01	0.1 to 0.2
0.02	0.1 to 0.2
0.06	0.3 or more

Thus all of the results obtained with a simple antigen, either by the passive or by the active method of sensitization, confirm the results obtained by the use of horse serum. It is evident, therefore, that the complex character of the antigen can in no way explain the result.

#### *Desensitization of the isolated organ*

It might be suggested that the results obtained in the living animals might possibly be explained on the following basis. It might be assumed that certain of the sensitized tissue cells have a relatively high avidity for antigen, and that these cells appropriate the first available fractions thereof, namely, the desensitizing dose. This would leave in reactive condition only the less avid antibodies, which would naturally require a higher dose of antigen to produce shock. The theory is unsupported by any known facts and is opposed by the observation that partially desensitized animals die with exactly the same anaphylactic symptoms as do the controls. It appears, moreover, to be definitely overthrown by the results which are obtained with the isolated sensitized guinea-pig uterus.

In a previous paper (3) (Study IX; The relation between partial desensitization and the minimal lethal dose in anaphylaxis) an attempt was made to study the quantitative alterations in the reactivity of the isolated uterus muscle after partial desensitization. This method has the advantage that it permits of the analysis of the phenomenon under simpler conditions, since possible complications, due to the presence of antibodies in the

blood or in the other cellular tissues, are at once eliminated. As a result of this study it was found that "an isolated uterus which has been partially desensitized by the addition of small amounts of antigen fails to respond except upon the administration of very large amounts of antigen." This conclusion is well illustrated by the following figure.<sup>1</sup>

It appears, therefore, that the isolated organ is affected by partial desensitization in exactly the same fashion as is the living animal.

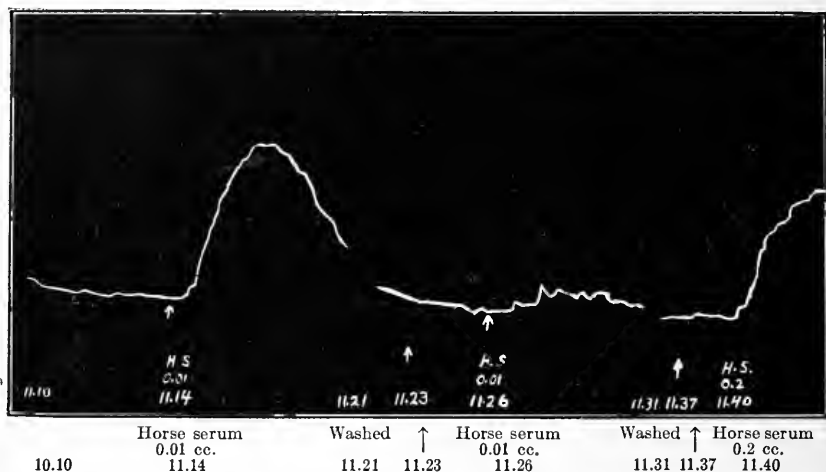


FIG. 1. GUINEA-PIG ACTIVELY SENSITIZED AGAINST HORSE SERUM, NOVEMBER 21

Killed December 12, and uterine tracing taken. After preliminary additions of antigen, the organ responds only to large amounts of the latter.

#### DISCUSSION

The results obtained upon the living animal, with horse serum as antigen, and with the method of passive sensitization, have been duplicated under other experimental conditions, calculated to test their validity. The use of a chemically simple antigen, of active sensitization, and of the isolated uterine muscle have failed to modify those results in any particular. It is evident,

<sup>1</sup> Reproduced from Proc. Soc. Exp. Biol. and Med., 1914, 11, 89.

therefore, that they do not depend upon any accidental features of technique, but are of general significance in anaphylaxis. Such being the case, it remains to discuss them not only in their bearing upon the problems of anaphylaxis, but with reference to immunological reactions in general.

*Discussion of results with horse serum*

In order to facilitate reference in the course of the following discussion, all the results obtained in the first series of experiments have been collected in the following table.

TABLE 22  
*General summary of tables 3 to 17*

GUINEA-PIG SERIES	(A)	(B)	(C)	(D)	(E)	(F)
	Sensitizing dose (Serum 265)	Minimal anaphylactic dose without desensitiza- tion (Horse serum)	MINIMAL ANAPHYLACTIC DOSE AFTER PARTIAL DESENSITIZATION			
			Desensitizing doses of horse serum			
			cc. 0.005	cc. 0.01	cc. 0.02	cc. 0.05
1	0.05	0.05		1.0 to 2.0		
2	0.075	0.03				
3	0.1	0.003		0.6		
4	0.2			0.6		
5	0.3	0.005	0.1	0.5	0.5	0.5

The table demonstrates, *first*, the very great increase in the minimal anaphylactic dose after partial desensitization.

*Secondly*, it establishes the fact that the final minimal anaphylactic dose is not the resultant of a quantitative relationship between the sensitizing dose of serum and the desensitizing dose of antigen. Thus in column D, the minimal anaphylactic dose after desensitization with 0.01 cc. of horse serum is almost exactly the same, namely, 0.5 cc. to 0.6 cc., whether the sensitizing dose of serum 265 is 0.1 cc., 0.2 cc., or 0.3 cc. Again, on line 5, the minimal anaphylactic dose after desensitization with 0.3 cc. of serum 265 is exactly the same, namely, 0.5 cc., whether the desensitizing dose of horse serum is 0.01, 0.02, or 0.05 cc. It is clear, therefore, that *antigen does not depress the reactivity of cellular antibody in regular quantitative ratios*. It is indeed true that differences may be discernible in the effects of

the desensitizing doses when the amounts of the latter are widely varied, as in columns C5 and D5. This observation, however, does not alter the terms of the conclusion above italicized. It merely indicates that the modification of reactivity of the cellular antibody is to some extent dependent upon the amount of antigen in combination therewith.

*Thirdly*, it illustrates a fact already fully discussed on page 476, namely, the impossibility of accounting for the increased anaphylactic dose after partial desensitization upon the theory that it represents the reaction of the free and unbound fraction of the cellular antibody. Were this the case, the minimal anaphylactic dose would necessarily lie between the figures 0.005 and 0.05, which are the limits of the minimal anaphylactic dose for the undesensitized animal in these experiments; as a matter of fact, the ascertained amounts are enormously higher. Therefore some other factor must be discovered to account for the increase in the minimal anaphylactic dose after partial desensitization.

The central feature of these conclusions is the very marked increase in the minimal anaphylactic dose after partial desensitization. Inasmuch as this condition cannot, as has been shown on page 476 be due to the neutralization of a portion of the antibody, leaving a residue free to react, there remains only one alternative explanation, namely, an altered and diminished condition of reactivity of the antibody as a whole towards fresh antigen. The diminished reactivity might, indeed, conceivably be due to a change in the cell itself, in virtue of which the latter would respond less energetically to an equivalent anaphylactic stimulus. That this explanation is incorrect, however, has been shown by experiments reported by Coca and the writer (4). Animals which had been sensitized toward two different antigens, and which had then been desensitized to one of them, responded to practically the same minimal anaphylactic dose of the second antigen as before. Hence it is clear that desensitization in no way affects the reactivity of the cell itself. Such being the case, the conclusion must be drawn that partial desensitization lowers the reactivity of the entire antibody content of the cell. The evidence of this lowered reactivity is the



enormous increase of antigen requisite to produce a reaction on the part of the cell. *Inasmuch as the antibody as a whole is altered, we must assume that it combines as a whole with the antigen.*

The conclusion that the cellular antibody as a whole unites with the desensitizing dose of the antigen permits us to advance to a more important problem, namely, a discussion as to the combining capacity of cellular antibody. It has been shown that partially saturated antibody possesses the capacity to react with fresh increments of antigen, and this can only be interpreted to mean that cellular antibody can unite with varying quantities of antigen. In other words, the combining proportions are not constant. A study of line 5, table 22, proves that this inference is correct, although it presents data of quite a different character. An animal sensitized with 0.3 cc. of the immune serum, and desensitized with 0.005 cc. of antigen, will not react to less than 0.1 cc. of antigen; whereas an animal similarly sensitized, but desensitized by 0.01 cc. of antigen, requires 0.5 cc. of the latter to cause a reaction. This proves that in the latter case the cellular antibody had bound considerably more of the larger desensitizing dose than it had of the smaller, since only on this basis can the difference in reactivity be explained. So that here again the evidence seems to show conclusively that *cellular antibody as a whole is capable of uniting with varying amounts of antigen.* This inference, it may be pointed out, is entirely independent of the previous conclusion as to the *altered energy* of reaction after partial desensitization.

Although the variability of combining proportions and the varying energy of reaction are, indeed, independent facts, they may to a certain extent be correlated. We must conceive of cellular antibody as capable of reacting with antigen in varying proportions, and with a degree of energy roughly dependent upon the amount of antigen already bound by it. If it has taken up a maximum amount of antigen, it fails to react with any further increment thereof, and the cell is absolutely desensitized. If it is free of antigen, it manifests a high avidity even for minute amounts of that substance. If it has already bound antigen in amounts insufficient to produce complete desensitization,

it is still capable of reacting with fresh antigen, but with greatly diminished avidity.

It is apparent that these relationships between cellular antibody and antigen are quite different from those which obtain as between precipitin and precipitinogen. In place of the strictly constant and quantitative relationships of the latter type of reaction, there is a wide range of variability. In view of the demonstrated identity of precipitin with sensitizin, this difference is a surprising and unexpected observation. The only available explanation seems to be an alteration in the character of the antibody, produced by the living cell of which it has become a part. Previous study (5) has demonstrated the fact that antibody when taken up by the cell becomes endowed with a highly increased avidity for antigen. The altered reactivity constitutes a second, and perhaps even more fundamental transformation. In neither case is it possible to penetrate the mechanism of the change. The results of cellular activity, but not the intimate processes themselves, are at present available subjects of scientific study.

The fact that cellular antibody that is already partly associated with antigen requires a large increment of the latter in order to produce an anaphylactic reaction, affords an explanation for certain well known phenomena. For example, relatively large amounts of antigen are required to produce an anaphylactic reaction if the intraperitoneal route is employed; it seems highly probable that the absorption of the first minute fractions of the antigen thus given serves partially to desensitize the cells, so that a very considerable additional amount must be absorbed before a reaction is induced. It seems likely, also, that this mechanism must help to protect the cells against violent anaphylactic reactions in infectious diseases. The bacterial protein of the disease is probably produced in gradually increasing amounts, which constantly fortify the antibodies built up by the cell against the anaphylactic effect of the later and larger accumulations of the same material. The body is, therefore, to a certain extent, safeguarded by this mechanism against some of the untoward consequences of its own defensive efforts.

## II. THE MODIFICATION OF INTRACELLULAR ANTIGEN BY ANTIBODY

There exists at the present time only one method for following the history and fate of foreign protein after it has once been taken up by the cells of the body. This method consists in the use of an immune foreign serum, the presence of which in the cell can always be determined by the reactivity of that cell to the specific antigen—in other words, the method of passive sensitization. It is a well known fact that an animal passively sensitized by means of the injection of a foreign immune serum gradually loses the hyper-susceptibility so conferred, the period of time during which it persists being determined by the amount originally injected, and varying from six days to more than two weeks. By the end of this period the foreign antibody has been destroyed and the induced sensitization is lost. Previous studies (6) have led to the belief that this destruction is brought about through the activity of the antibody produced by the cell in response to the presence of the foreign protein. Inasmuch as the latter are antibodies, the former have been called anti-antibodies. In the present paper an attempt is made to determine the effect exerted by this native anti-antibody upon the foreign antibody. The method of experimentation consists in ascertaining the mode of reaction of the foreign antibody in the presence of native anti-antibody, as compared with its reaction in the normal animal, not possessed of such anti-antibodies.

A series of guinea-pigs received an intraperitoneal injection of 0.1 cc. of normal rabbit serum, in order to stimulate their cells to the production of antibodies toward rabbit serum. It has been shown that such antibodies are capable of neutralizing the antibodies of an immune rabbit serum (6). After the lapse of a sufficient interval of time, these animals were passively sensitized by the injection of the serum of a rabbit immunized against horse serum. Upon the following day these animals received intravenous injections of horse serum in graded amounts, in order to determine the minimal anaphylactic dose. For comparison, the minimal anaphylactic dose was also determined in a series of guinea-pigs similarly sensitized but not previously injected with normal rabbit serum.

TABLE 23

*Guinea-pigs weighing from 210 to 220 grams, injected intraperitoneally with 0.1 cc. of normal rabbit serum. Thirteen days later, passively sensitized by the injection of 0.6 cc. of serum 265 (rabbit versus horse serum) intraperitoneally. On the fourteenth day injected intravenously with graded amounts of horse serum. (The minimal sensitizing dose of serum 265 was 0.05 cc.)*

GUINEA-PIG	HORSE SERUM INTRAVENOUSLY	RESULT
	cc.	
1	0.4	Moderate symptoms
2	0.6	Severe symptoms; died in two hours
3	0.8	Severe symptoms after six minutes; recovered
4	1.0	Died in 8 minutes
5	1.2	Delayed, mild symptoms
6	1.2	Died in four minutes
7	1.5	Died immediately
8	1.8	Died immediately

In this table no animals receiving less than 1 cc. of horse serum died in acute anaphylaxis. Of those receiving more than 1 cc., all except one gave the typical result. The series is not perfectly regular owing to the fact that the animals doubtless possessed somewhat varying amounts of anti-rabbit serum immune bodies, in spite of the fact that the preliminary treatment had been uniform. The minimal anaphylactic dose in normal animals passively sensitized by the injection of 0.6 cc. of serum 265 was found to be 0.006 cc. of horse serum.

A number of other series were treated in similar fashion, although the sensitizing doses and the intervals were somewhat varied. Essentially, the results were similar to those in table 23. It was regularly found that the minimal anaphylactic dose was very much greater than in the normal control series.

A similar set of experiments was carried out with the serum of a rabbit immunized against crystalline egg albumin, in order to determine whether the use of a complex antigen, such as horse serum, could in any way be responsible for the result. The data obtained in this series are presented in table 24.

The minimal anaphylactic dose in animals passively sensitized by 0.4 cc. of this rabbit's serum, but not previously injected with

TABLE 24

*The animals were prepared as for the preceding experiment, but were passively sensitized on the fourteenth day by the intraperitoneal injection of 0.4 cc. of the serum of a rabbit immunized against crystalline egg albumin. The toxic injections were made on the following day*

GUINEA-PIG	5 PER CENT CRYSTALLINE EGG ALBUMIN, INTRAVENOUSLY	RESULT
	cc.	
1	0.05	No symptoms
2	0.1	Mild symptoms
3	0.2	Severe symptoms
4	0.3	Immediate death
5	0.4	Immediate death

normal rabbit's serum, was 0.002 cc. of the egg albumin. The table shows the same increase of the minimal anaphylactic dose as was observed in passive sensitization to horse serum.

#### *Discussion on the modification of cellular antigen*

The fact is therefore established that animals which possess anti-antibody, and are then passively sensitized with immune serum from the corresponding alien species, respond to antigen only in relatively enormous amounts. To this may be added the further fact, previously established (6), that such animals require relatively large doses of immune serum to become passively sensitized. It has also previously been shown that cellular anti-antibody, when present in relatively small amounts, merely lowers the reactive energy of alien antibody, while in larger amounts it may completely abolish the latter. *The important point is that such alien antibody when altered in its reactivity, presents a true change of character, a qualitative transformation.* Thus, in the experiments reported, it required 1 cc. of horse serum to produce a typical anaphylactic response with partially neutralized antibody. Antibody in a normal animal, however, no matter in what amounts it has been given, never reacts in this manner. If given in minimal sensitizing doses, it responds to 0.05 cc. of antigen; if in larger doses, to 0.006 cc. Therefore the partially neutralized antibody must be held to be entirely different from simple antibody, as regards its reactivity.

The explanation of such an effect depends upon the same principle as that invoked in the first set of experiments. It is clear that the anti-antibody does not simply destroy a certain portion of the antibody, leaving the remainder free to react. Such an explanation is incompatible with the facts of the case, inasmuch as the altered antibody does not react quantitatively like a smaller amount of unaltered antibody. We must assume a union of the two factors in toto, resulting in a lowered reactivity of the entire antibody. Bordet has suggested this explanation for analogous conditions involving either hemolysins or toxins. The point of view might be summarized by saying that anti-antibody does not simply reduce the amount of antibody; it depresses the functional activity of its ergophore group, whether this group manifests its activity as hemolysin, sensitizin, or toxin.

The altered reactivity of antibody may be described as an "attenuation." It is evident that such attenuation must be of great service to the organism as a protection against the effects of toxins. It seems clear from many previous studies that these substances are not actually destroyed, or, if so, only very slowly, by the antitoxins; they are, however, at once deprived of their toxic properties. From the results of the present experiments it is safe to conclude that the same sort of process occurs within the cell, thereby materially enhancing its protection, in the course of its attempt to dispose of toxins.

#### *General theoretical considerations*

The conclusions reached in the preceding discussions permit of a fairly definite conception as to the mode of interaction of antigen and antibody within the cell. It is the purpose of the present section of this paper to indicate the relationship of these conceptions to the other known data of immunology.

The essential features are as follows:

1. Cellular antibody combines with antigen in varying proportions, and its reactive energy is thereby impaired.
2. Cellular antibody lowers the functional activity of cellular antigen—depresses its so called ergophore group.

Combination of antigen with antibody in varying proportions has been observed chiefly in connection with the neutralization of toxins by antitoxins. The well known Danyz-Dungern phenomenon establishes the fact that a given quantity of antiricin, or of diphtheria antitoxin, is capable of going into complete union with varying amounts of their respective antigens. Evidently this reaction is of a type quite different from the strictly quantitative relations which exist in the precipitation reaction. Bordet and others have attempted to interpret all immunological reactions as colloidal phenomena, a view largely based on the common characteristic of combination in varying combining proportions. More exact analysis of the precipitation reaction has, indeed, invalidated this particular aspect of the comparison. It still obtains, however, for certain other immunological reactions, as above stated, namely those between toxins and antitoxins, and especially those which take place within the cell. Now, although it is undoubtedly true that this type of reaction constitutes a serious objection to Ehrlich's conception of immunological reactions, which postulates combining groups and constant proportions, there are equally valid grounds for questioning the analogy with adsorption or surface reactions. Bordet (7) himself concedes that specificity is a feature which fundamentally upsets this analogy, an admission which Landsteiner also makes. It is perhaps best, therefore, simply to record the fact of combination in varying proportions, and not to attempt at present to classify it rigidly in the available categories of either physics or chemistry, although it unquestionably seems to be more closely allied to the former.

The fact that one and the same antibody, namely precipitin, depending upon whether it is free in solution or is bound to the cell, may conform to either the chemical or the physical type of reaction, suggests the possibility that these types may not, after all, be so radically different as would appear. It is of interest to remark that just as precipitin may become bound to the cells as sensitizing antibody, so undoubtedly it exists as cellular antibody before being thrown off into the blood; thus the transformation of the type of reactions seems to be reversible. This

fact suggests another analogy which, though new, is perhaps no more valid than those previously condemned. Many substances are known which may exist either in simple or in colloid solution; such are arsenic trioxide and iron hydroxide. When in simple solution, these substances react according to chemical laws, whereas in colloidal solution they have the properties of colloids. This analogy breaks down, however, in a crucial point. If to the colloidal solution is added a substance with which it has a chemical affinity, the reaction proceeds not as a surface reaction, according to colloidal laws, but along chemical lines, on a molecular basis. It is just this one essential detail in which antibody is peculiar. In solution it reacts with its antigen quantitatively; attached to the cells, it reacts with the same antigen in varying proportions, like a colloid.

The fact that partially combined cellular antibody will unite with additional antigen only in case the latter is present in great excess, appears to be unique in immunological science. As a matter of fact, however, there is no available method except that of anaphylaxis for determining such a condition. In the Danysz-Dungern experiment, for example, it is quite possible that the bound antigen impairs the avidity of the antibody towards fresh increments of toxin, but, if so, there is no means of demonstrating the fact.

The second feature of interest is the attenuation of the cellular antibody by the anti-antibody. This behavior of cellular antibody is entirely analogous to certain other immunological phenomena as observed in the test tube. Thus, if hemolysin be treated with anti-hemolysin, depending on the relative amounts, the hemolytic power may be either entirely destroyed, or qualitatively altered. Bordet (8) has discussed this question with his usual lucidity, as follows.

We must distinguish very carefully in the effects of a poison between quality and quantity. I may recall the observations which I offered in 1903, that showed that a hemolysin may manifest toxicity in two different ways; when employed in a small dose without being in any way affected by an antitoxin (antihemolysin), it hemolyzes a small amount of corpuscles rapidly, but owing to the fact that it is not present in



sufficient quantities it cannot destroy many of them. On the other hand, a large dose of the same hemolysin to which a little of its antitoxin has been added and which has been transformed thereby, according to my idea, into an incompletely saturated complex, can hemolyze a large amount of corpuscles but produces hemolysis *very slowly*, even if a very small amount of the corpuscles is added. It would seem, then, as if such a mixture included a strong dose of poison (the hemolysin) the activity of which is distinctly depressed owing to the addition of a little antitoxin, and the result of the experiment is in no way compatible with the idea that such a mixture contains, in addition to perfectly neutralized hemolysin, a certain excess of perfectly intact hemolysin.

The same sort of observation has been made of the effects of diphtheria antitoxin upon toxin. These relations are summarized by Bordet (9) in the following words.

When to a given amount of toxin, antitoxin is added in an amount that does not suffice completely to neutralize it, the antitoxin molecules are not monopolized by certain of the toxin molecules whose affinities become thereby satisfied, leaving the remaining toxic units intact. On the contrary, the antitoxin molecules are shared by, and divided equally among, all the toxic molecules present, which thenceforth are partially saturated and lose to a certain extent their original toxicity. The phenomenon of intoxication caused by injecting this compound into animals may not be the same as those produced by a mixture of neutralized toxin plus intact toxin.

Evidently these relations are identical with those disclosed by the present series of experiments.

The effects upon each other of cellular antigen and antibody have been described but have not been explained. How are we to conceive of their mutual relationships? A very common theory maintains that the antigen undergoes a sort of digestion, a process in which the complement acts as proteolytic ferment, and the antibody, also known as amboceptor, serves as a bond to unite these two substances. In support of this theory, we have the observed fact that mixtures of antigen and antiserum, when incubated, present definite chemical evidence of protein decomposition. Recent studies, however, have shown that it

is not the antigen, but the antiserum itself, which undergoes this change; moreover, the proteolysis is in no way a consequence of the antigen-antibody reaction in the mixture, but takes place in the same fashion when two indifferent normal sera are mixed. On the other hand, there is much evidence which seems incompatible with the digestion theory. When precipitate, formed through the interaction of antigen and antiserum, is redissolved, the antigen may be recovered unchanged from the fluid. In the same manner, the complex formed by the interaction of diphtheria toxin and antitoxin may by proper methods be dissociated, yielding the toxin in unchanged condition. These facts indicate that *in vitro* the two components of an immunological couple go into combination and coexist for a long time unaltered, and that the antibody does not digest, or otherwise destroy the antigen. Eventually, however, these complexes tend to become firm and indissociable. Such data as are at present available concerning the intracellular disposal of antigen points to a similar conclusion. I have previously found that a guinea-pig passively sensitized with a considerable excess of an alien immune serum requires a period of two weeks or more to neutralize the anchored, or cellular antibodies, and that during the second week of this period, as the native anti-antibodies accumulate, there is definite evidence that both of these substances coexist in the cell. These findings have been confirmed and amplified by Denzer (10). If, however, we were to judge by the analogy of ferment actions, we should have expected that the excess of intracellular anti-antibodies, would lead to a rapid and complete destruction of the foreign antibodies. The experiments recorded in the present paper can also only be interpreted on the theory that antigen and antibody coexist in the cell. The marked alteration of reactive energy which both exhibit is not explicable, as has been repeatedly pointed out, on the theory of partial destruction. It suggests strongly some sort of physical effect, and this is quite in harmony with the other colloidal features characteristic of the interaction of these substances in the cell. But exactly what type of physical condition is responsible for this diminution in reactive energy is entirely a matter of speculation.

It might be asked how, upon the colloid theory, we are to explain the eventual fate of the antigen. As a matter of fact, we possess no data relative to this problem. Antigen ceases to be demonstrable in the cell when its functional activity is completely neutralized. Thus sensitizing antibody gives evidence of its presence only through its reactivity with antigen. There is, however, no good reason to believe that it ceases to exist in the cell simply because it no longer reacts. The functional activity of precipitin in the test tube may be destroyed by heat or other agency, yet the antibody can still be demonstrated by other methods. At the present time our methods do not permit us to identify foreign antibody in the cell after it has ceased to react; its further history and eventual disposition are, therefore, obscure and entirely hypothetical.

#### SUMMARY

##### *Experimental observations*

##### A. Neutralization of cellular antibody by antigen

1. When guinea pigs are passively sensitized by the injection of rabbit immune serum, quantitative studies show that the *anchored, or cellular, antibody is capable of combining with varying quantities of the antigen*. This observation holds true whether the antigen be complex, such as horse serum, or simple, such as crystalline egg albumin. It holds true whether the experiments are conducted on the living animal, or on the isolated uterus. Moreover, it holds true, also, of native antibody, as shown by experiments in active anaphylaxis.

2. Partially combined cellular antibody manifests a marked diminution in its affinity for fresh antigen. This diminution is inverse, but only very roughly, to the degree of saturation by antigen. Considerable variation in the amount of the desensitizing dose of antigen may produce in practically the same degree a loss of reactivity, or of avidity, toward fresh increments of antigen.

3. The minimal anaphylactic dose after partial desensitization

shows an enormous increase over that in the undesensitized animals. This increase cannot possibly be accounted for on the theory of neutralization of part of the cellular antibody, leaving the remainder free to act.

4. Partially saturated antibody shows not a diminished, but a qualitatively altered reactivity.

#### B. Neutralization of cellular antigen by antibody

5. Native guinea-pig anti-antibody attenuates alien (rabbit's) sensitizing antibody. In relatively large amounts the former may completely abolish the reactivity of the latter; in smaller amounts it lowers the reactivity in such a fashion that very large amounts of antigen are required to induce an anaphylactic response.

6. Partially neutralized antigen shows, not a diminution, but a qualitative alteration of its reactive function.

#### *Theoretical considerations*

7. The combination of cellular antibody with antigen in varying proportions suggests an analogy with colloidal reactions, or adsorption phenomena. A very essential point of difference is the specific affinity of the two reagents.

8. The Danyz-Dungern phenomenon illustrates the fact that toxins and antitoxins in vitro likewise combine in varying proportions.

9. The alteration of the reactive capacity of cellular antibody by anti-antibody also suggests the analogy of colloidal reactions. It is entirely similar to the effect of diphtheria antitoxin upon diphtheria toxin.

10. The same antibody, when in solution as precipitin, combines with antigen quantitatively, and in strictly constant proportions, to form precipitate; when attached to the cell, as sensitizing antibody, it combines with antigen in varying proportions. The living cell, therefore, modifies its properties.

11. There is no general law governing the mode of reaction of antibody. Depending upon circumstances, it may combine with antigen according either to chemical or to physical (colloidal) analogies.

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## STUDIES IN PROTEIN INTOXICATION<sup>1</sup>

### II. VASCULAR LESIONS IN CHRONIC PROTEIN INTOXICATION

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The literature contains but few references to vascular lesions in acute anaphylaxis, and, so far as I can find, only one reference (7) to vascular lesions produced by repeated injections of foreign proteins. Gay and Southard (1) studying the lesions produced in guinea pigs in acute anaphylaxis, report finding fatty changes in capillary endothelium which they interpret as being the cause of the hemorrhages which are so constantly found in acute anaphylaxis. Rosenau and Anderson (2) were unable to find these lesions in acute anaphylaxis, but Longcope (3) and others mention finding them frequently. Longcope (3), (4), (5) in studying the histologic results of repeated injections of foreign proteins in various animals, found marked, and in some cases, even severe lesions in the parenchymatous cells of heart, liver, and kidney, with varying degrees of fibrosis, but, aside from perivascular infiltration, found no arterial lesions. Stoddard and Woods (6) produced nephritis in rabbits by repeated injections of Vaughan's protein poison, but were not able to find lesions of arteries. The author (7), in studying the lesions produced in the kidneys of guinea pigs by repeated injections of foreign protein, noted very distinct lesions of a degenerative type in the smaller arteries. These lesions consisted in a swelling of the arterial wall, with, in some cases, very marked degeneration of the intima, and vacuolation and fissuring of the intima and

<sup>1</sup> The first paper of this series appeared in vol. I, no. 1, of this journal under the title: Kidney lesions in chronic anaphylaxis.

media. Sometimes there was a marked proliferation of the endothelium. This change involved nearly all the vessels in any section in which it occurred. It was rarely seen in the larger arteries. These lesions were found in a majority of the experimental animals, but were less frequent in those animals receiving no injections for several weeks before being killed. The lesions were not seen in animals dying after one or two shocks, nor in normal controls.

The present work is a continuation of the work previously reported, with especial reference to the occurrence of vascular lesions in various organs.

#### METHODS

Thirty guinea-pigs were used in this study, including six normal controls, and five controls that were injected repeatedly with protein, but at such short intervals that they failed at any time to develop anaphylaxis. The materials used and the methods of injecting were the same as those previously described (7), the object desired being to give each animal as many anaphylactic shocks as possible during the course of the experiment, using egg-white and beef serum. Some of the animals received as many as twenty injections of protein, and some were under observation for longer than eight months. Those animals that died were posted at once, and others were killed at varying intervals up to eight weeks after the last injection. Sections were cut from heart, lungs, liver, kidney, spleen, and aorta.

#### RESULTS

Arterial lesions were observed in all the organs studied except the lungs, but were most frequent and most marked in the liver. The lesion apparently begins in the intimal coat. The first change observed is a swelling or edema, and later a granular degeneration and vacuolation of the cytoplasm of the endothelial cells somewhat similar to the vacuolation and degeneration previously described as occurring in the tubular epithelium of the kidney (7). Sometimes fissuring of the intima is observed.



The nuclei may disappear from the endothelial lining. No thromboses have been observed, so this stage is quickly succeeded by a stage of regeneration. The nuclei are greatly increased over what is normal for an artery of a given size, and are round instead of oval when the artery is viewed in cross section. Edema is noted about the internal elastic lamina, so that the lamina may be rendered quite prominent, and it is frequently swollen, and occasionally it is split or frayed. J. W. McMeans (8) discusses splitting of the elastic fibers of arteries, and comes to the conclusion that this is a characteristic early change in arteriosclerosis. Frequently the swelling and edema described in the intima extend also to the media, especially the inner portion of it, and there is a disappearance of cell detail, with occasionally an extension of the fissuring noted in the intima. No distinct proliferation of connective tissue was observed, and no changes that were definitely chronic in character.

The most severe lesions are found in animals dying in shock, or killed within one week from the time of the last injection. In animals killed five weeks or longer after the last injection the lesions were still present, and frequently well marked, but were less severe than in the first group. The arterial lesions occurred in 100 per cent of the livers of experimental animals, and were characterized as "severe" in 37 per cent, "moderate" in 44 per cent, and "mild" in 19 per cent. The lesions were found in the kidneys of 66 per cent of the experimental animals. Of the animals dying of shock, or killed within 2 weeks after the last injection, 83 per cent showed lesions, and of the animals killed from three to eight weeks after the last injection, 50 per cent showed lesions. Of the lesions in kidneys, 17 per cent were characterized as "severe," 50 per cent as "moderate," and 33 per cent as "mild." Lesions were present in 66 per cent of the hearts, and were not severe in any case. Of these lesions, 66 per cent were characterized as "mild." Lesions of the endocardium were not observed in any case. In only five of the spleens studied were arteries found which were large enough to show the lesions. Of these arteries, however, all showed the characteristic changes, 40 per cent to a "mild" degree, and 60 per cent to a "moderate" degree.

The aortas in this series were practically without pathological change, only one showing a slight edema beneath the internal elastic lamina. The vasa vasorum of the aortas appeared normal.

Perivascular infiltration was found in 60 per cent of the experimental animals. It was usually not very severe. It was most common and most pronounced in animals dying in shock, or killed within 5 weeks after the last injection. It was not observed in any animals killed more than five weeks after the last injection, though the lesions of the intima were often quite distinct in these cases.

Lesions of the type here described were found chiefly in the smaller arteries. They were rarely found in the larger arteries, and not at all in the capillaries (except the lesions of the capillary loops of the glomerular tufts of the kidney previously described (7) ). The lesions were practically absent from veins.

Five animals were examined that had received from twelve to seventeen doses of protein during a period of from six weeks to four months. The total amount injected ranged from 16 cc. to 36 cc. of undiluted egg-albumin, and in one case 56 cc. of beef serum. The doses were so spaced as to avoid anaphylactic shock at any time. The character and distribution of the lesions in this group were similar to those of the anaphylactic group, except that they were less numerous, and much less severe. Vascular lesions were not seen in the hearts nor in three of the kidneys. In the two remaining kidneys the lesions were slight. Vessel changes in liver and spleen were slight or moderate. Perivascular infiltration was not observed in any case. This, in a general way, is in accordance with Longcope's (9) findings in animals receiving a single large dose of protein, and allowed to live for several weeks. He obtained lesions which were similar in character to those occurring in reinjected sensitized animals, but were much less severe, and less frequently observed. Apparently the splitting of protein in the body tends to produce certain types of lesions, whether that splitting is sudden and explosive, as in the case of anaphylactic shock, or slower, as in the case of unsensitized or refractory animals. The severity of the lesions depends to a certain extent upon the severity of the symptoms which follow the protein injection.

That the lesions are more severe in the liver than in the other organs studied may be due to the fact demonstrated by Manwaring (10) that the liver is the most important organ concerned in the production of anaphylaxis.

In studying the tissues of this series, one is struck with the similarity of some of these lesions to post mortem changes. This is particularly true of the edema of both intima and media, and the vacuolation of the intima. An apparently confirmatory circumstance is the fact that the lesions are most noticeable in the animals that died as the direct result of one particular injection, and often these animals died in the cages and were not discovered till the next day, whereas the lesions were least marked in those animals that were allowed to live for several weeks, and were killed and posted at once. On the other hand the increase in the number of endothelial nuclei is certainly not post mortem, and is marked in 40 per cent of the animals studied. When the animals are classified according to the greatest amount of time that might possibly have elapsed between the time of death and of post mortem examination, the "moderate" and "severe" lesions occur with practical uniformity in all of the groups. If the microscopic appearances were due to post mortem changes, we should expect to find the least marked changes in those animals killed and posted at once, and the most marked changes in those animals in which a period of several hours elapsed between death and post mortem. The "mild" lesions occur only in those animals allowed to live for five to eight weeks after the last injection before being killed, but the mildness of the lesion here might be due as well to the fact that these animals had had no recent anaphylactic shock, as to the fact that they were posted soon after being killed.

The literature on post mortem changes is not helpful in the present instance, as the experiments have been done with organs removed from the body and kept in salt solution or serum at varying temperatures, and it is quite possible that the changes might be different from those occurring in the intact animal body. Wells (11) however, states that endothelium is the most resistant tissue to post mortem change. In order to put the matter to a definite test four normal pigs were killed with

chloroform, just as the experimental animals had been, and kept under various conditions corresponding to the conditions under which the experimental pigs might have been kept before being posted. One was opened immediately and pieces of tissue were removed and fixed, as a control. This one was then frozen and thawed, and tissues were removed again. Others were kept at room temperature (20°C.) and at 30°C. for twenty-two hours and then frozen, and one was kept at 5°C. for four days. When tissues were removed more than once from the same animal, as small an incision as possible was made, the organs were handled as little as possible, and the incision was carefully closed in order to simulate as nearly as possible what takes place when a dead animal is left in the cage over night, and then placed in the refrigerator, and perhaps frozen before being posted. It is probable that these tests covered all of the possible conditions under which animals of this series may have been kept before being posted. Sections were studied from the most important organs. None of the animals in this control series showed more than very slight post mortem changes, which could be easily recognized as such, indicating that the experimental series were really appropriate for study. None of the animals in this control group showed any of the changes that have here been considered as characteristic of protein intoxication.

It is necessary to guard, also, against spontaneous lesions. In some of the animals studied, including some of the normal controls, arteries were found whose walls showed apparent thickening of the media, but no changes were seen in the intima of arteries of any of the normal controls. Consequently in studying this series all changes in the media were discounted unless they were associated with changes in the intima as well. It seems justifiable to assert that the lesions here described cannot be explained as due either to post mortem or to spontaneous changes.

#### DISCUSSION

There is no good evidence that the vascular lesions are themselves responsible for the lesions found in the parenchymatous

cells, but, considering the number of types of tissue that are affected—epithelial cells of kidney and liver, muscle cells of heart, connective tissue of all of these organs, endothelial cells of blood vessels—it is more probable that we are dealing with a poisonous substance of rather general affinities. The failure to produce distinctly chronic lesions of blood vessels is probably related to the readiness with which guinea-pigs become refractory to the injection of foreign proteins, though it might possibly indicate that the reparative power of endothelium is great, or that the toxic substance is not highly toxic for all of the vascular tissues. The relation of these results to the question of clinical arteriosclerosis is problematical, though the increasing list of human diseases which are being interpreted as due, at least in part, to anaphylaxis makes this question of great importance. The practical absence of lesions in veins, and also in capillaries (except as previously noted in the capillary loops of the glomerular tufts of the kidney) is interesting. The capillary lesions described by some of the earlier writers as following acute anaphylaxis were seen particularly in the stomach, intestine, and lung, and not in those organs in which later writers have noted parenchymatous lesions: kidney, liver, and heart. The evidence thus far at hand does not speak for a characteristic or specific histologic picture as a result of protein intoxication. The picture of chronic protein intoxication is quite different from that of acute intoxication, but probably the differences are quantitative rather than qualitative. The lesions produced by the injection of the protein poison do not seem to differ greatly from those produced by injections of foreign proteins in sensitized animals, though perhaps the number of experiments reported with the use of the protein poison is too small to make valid comparisons.

#### CONCLUSIONS

1. Repeated injections of foreign proteins in sensitized guinea-pigs produce lesions of the smaller arteries of liver, kidney, spleen, and heart, characterized by degeneration and regeneration of endothelium, edema and fissuring of intima and media, and

sometimes splitting of the internal elastic lamina. These lesions are interpreted as being subacute rather than chronic.

2. These lesions are rarely found in the larger arteries, and not at all in the aorta. They are practically absent from veins and capillaries.

3. These lesions occur in 100 per cent of the livers, 100 per cent of the spleens, 66 per cent of the kidneys, and 66 per cent of the hearts, and were most severe in the livers, and least severe in the hearts.

4. The lesions were most severe in animals dying, or killed soon after the last injection, and were less frequent and severe in animals killed several weeks after the last injection.

5. Perivascular infiltration was observed in 60 per cent of the animals, though never severe, but was not found in animals killed more than five weeks after the last injection.

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#### DESCRIPTION OF FIGURES

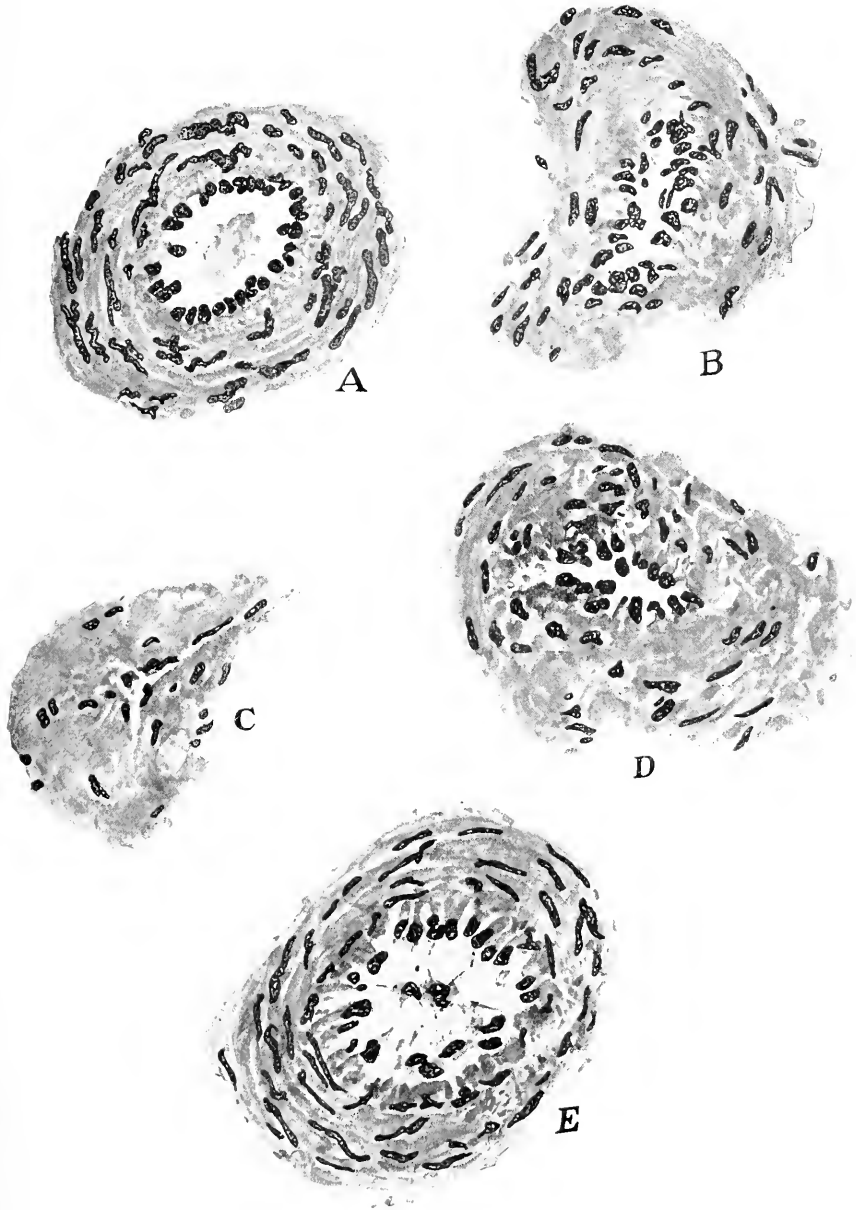
A. Guinea-pig 11, kidney. Marked increase of endothelial nuclei. Pig received twenty injections of egg-albumin. Killed eight weeks after last injection.

B. Guinea-pig 102, liver. Marked increase in endothelial nuclei, edema and fissuring of intima and media. Pig received seven injections of egg-albumin and seven of beef-serum. Killed eight days after last injection.

C. Guinea-pig 29, liver. Marked edema of arterial wall. Pig received five injections of egg-albumin and two of serum. Anaphylactic death.

D. Guinea-pig 24, liver. Increase of endothelial nuclei, edema and fissuring of intima and media. Pig received seven doses of serum. Anaphylactic death.

E. Guinea-pig 24, spleen. Edema and fissuring of intima. Localized edema of media.







# FATE OF THE FOREIGN PROTEIN IN THE ACUTE ANAPHYLACTIC REACTION

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The principal theories concerning the nature of the acute anaphylactic reaction can be grouped in two classes. Theories of the first class picture the reaction as due to an increased receptor apparatus in the fixed tissues. Those of the second class conceive it as a result of an increased proteolysis by the body fluids.

It was thought that evidence of the validity or non-validity of these theories might be obtained by applying perfusion methods to isolated organs and tissues (1).

*Material and methods.* Isolated liver, kidney, intestines, lungs and hind-quarters of normal, anaphylactic and immune guinea-pigs, rabbits and dogs. Perfusion fluids: dilute solutions of foreign proteins, either in Ringer's solution or in dilute defibrinated normal or anaphylactic blood. Perfusions made at 37°C., under a pressure and rate of flow approximately equal to the normal blood-pressure and blood-flow in the organ or tissues studied. Perfusion fluids freed from cellular elements by centrifugation, and changes in the concentration of the foreign protein estimated by titration with a specific precipitin.

*Precipitin titration.* 1 cc. 10 per cent precipitating serum, plus decreasing amounts of the fluid to be titrated, the volumes being made uniform (2 cc.) with NaCl solution. Readings made at the end of two hours at 37°C., and after twenty-four hours in the ice chest. In recording the results, the following symbols are used:

- +++ = Heavy precipitate
- ++ = Medium precipitate
- +
- tt = Heavy turbidity, no precipitate
- t = Distinct turbidity, no precipitate
- ? = Trace of turbidity, no precipitate
- 0 = No turbidity, no precipitate

The numbers at the top of the columns show the dilutions of the perfusion fluid in the precipitin tests, thus: 64 = dilution 1:64, etc. The tables record the twenty-four hour readings.

#### A. QUANTITATIVE CHANGES DUE TO THE ANAPHYLACTIC BLOOD

If 0.25 per cent to 1 per cent egg white or goat serum is added to 50 per cent defibrinated anaphylactic guinea-pig blood, and the mixture is incubated fifteen minutes, no decrease in the amount of the foreign protein is observed, when compared with a control mixture of the same protein in normal blood (table 1).

TABLE 1

##### *Quantitative changes in anaphylactic blood*

(a) 0.25 per cent goat serum in 50 per cent defibrinated anaphylactic guinea-pig blood; (b) 0.25 per cent goat serum in defibrinated normal guinea-pig blood; mixtures incubated fifteen minutes, corpuscles removed by centrifugation, supernatant fluids titrated with a specific precipitin.

FLUID	PRECIPITIN TITRATION									
	64	128	256	512	1024	2048	4096	8192	16384	32768
Anaphylactic blood.....	+++	+++	++	++	+	+	+	t	t	0
Normal blood.....	+++	+++	++	++	+	+	+	t	?	0

#### *Conclusion*

*The immediate humoral anaphylactic reaction (2) can not be looked upon as a hydrolytic cleavage or destruction of the foreign protein, sufficient to prevent its giving quantitatively the specific precipitin reaction.*

#### B. QUANTITATIVE CHANGES DUE TO THE FIXED TISSUES

##### *1. Perfusion of normal tissues*

If 0.1 per cent to 1 per cent egg white or goat serum in Locke's solution or Ringer's solution or in 25 per cent to 50 per cent defibrinated normal or anaphylactic blood is repeatedly perfused through the organs of normal animals, no decrease in the concentration of the foreign protein is observed, on subsequent titration with a specific precipitin (tables 2 and 3).

TABLE 2

*Blood-free perfusion of normal tissues*

Liver of 1500 gram normal rabbit, washed free from contained blood by a preliminary perfusion with 200 cc. Ringer's solution, then repeatedly perfused with 50 cc. 1 per cent goat serum in Ringer's solution.

FLUID	PRECIPITIN TITRATION									
	2	4	8	16	32	64	128	256	512	1024
Before perfusion.....	+++	+++	+	tt	t	t	0	0	0	0
After eight perfusions.....	+++	+++	+	t	t	t	0	0	0	0

TABLE 3

*Defibrinated-blood perfusion of normal tissues*

15 cc. 0.25 per cent goat serum in 50 per cent defibrinated anaphylactic guinea-pig blood, repeatedly perfused through the liver of 500 gram normal guinea-pig. Control sample kept at 37°C.

FLUID	PRECIPITIN TITRATION									
	32	64	128	256	512	1024	2048	4096	8192	16384
Control sample.....	+++	+++	+	+	+	+	tt	t	t	0
Perfused sample.....	+++	+++	+	+	+	+	+	t	t	0

*2. Perfusion of anaphylactic and immune tissues*

If the tissues of anaphylactic and immune animals are similarly perfused, they give results identical with those with normal tissues (tables 4 and 5).

TABLE 4

*Perfusion of anaphylactic tissues*

Liver of 1500 gram anaphylactic rabbit, repeatedly perfused with 50 cc. 0.5 per cent goat serum in 50 per cent defibrinated anaphylactic rabbit blood.

FLUID	PRECIPITIN TITRATION									
	2	4	8	16	32	64	128	256	512	1024
Control sample.....	+++	+++	+++	+	+	+	+	t	0	0
Perfused sample.....	+++	+++	+++	+	+	+	t	t	0	0

TABLE 5

*Perfusion of immune tissues*

Liver of 12 kg. immune dog, washed free from contained blood by a preliminary perfusion with 600 cc. Ringer's solution, repeatedly perfused with 100 cc. 0.25 per cent goat serum in Ringer's solution.

FLUID	PRECIPITIN TITRATION									
	2	4	8	16	32	64	128	256	512	1024
Control sample.....	++	++	++	+	+	+	t	t	0	0
After forty perfusions.....	++	++	++	+	+	+	t	t	0	0

Of special interest are the tests with the lungs of anaphylactic guinea-pigs. These, perfused with the specific protein, either in Ringer's solution, or in normal or anaphylactic blood, are thrown into typical anaphylactic reactions (2). The reactions, however, are not accompanied by a demonstrable decrease in the concentration of the foreign protein in the perfusion fluids (table 6).

TABLE 6

*Perfusion of anaphylactic lungs*

Lungs of 250 gram anaphylactic guinea-pig, washed free from contained blood by a preliminary perfusion with 20 cc. Locke's solution, perfused ten times with 10 cc. 0.25 per cent goat serum in 50 per cent defibrinated normal guinea-pig blood. Typical anaphylactic response.

FLUID	PRECIPITIN TITRATION									
	4	8	16	32	64	128	256	512	1024	2048
Control sample.....	++	++	++	++	+	+	+	t	0	0
Perfused sample.....	+++	++	++	++	+	+	+	t	0	0

*Conclusion*

*No demonstrable destruction or binding of the foreign protein by the fixed tissues takes place, during the acute anaphylactic reaction.*

## SUMMARY

Perfusions of isolated organs and tissues with dilute foreign proteins furnish no evidence of a measurable destruction of the

foreign protein by the blood serum, nor of an appreciable destruction or binding of the foreign protein by the fixed tissues during the acute anaphylactic reaction.

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## RÔLE OF HEPATIC TISSUES IN THE ACUTE ANAPHYLACTIC REACTION

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It was pointed out in 1910, that the pronounced fall of blood-pressure, which is the essential feature of the acute anaphylactic reaction in the dog, is not due to a direct action of the foreign protein on the sensitized blood-vessels, but is an indirect phenomenon, due to the explosive formation or liberation of depressor substances by the liver.<sup>1</sup>

This observation has led us to study the rôle of hepatic tissues in anaphylactic guinea-pigs, by the application of perfusion methods to isolated organs.

*Material.* 200 gram guinea-pigs sensitized by a single intraperitoneal injection with 0.025 cc. goat serum; tested from twelve to sixty days after the injection.

*Methods.* *a. Hepatic perfusion.* Afferent cannula in portal vein, delivering the perfusion fluid under constant pressure and temperature; ligation of remaining portal vessels and of vena cava below liver; efferent cannula in vena cava above diaphragm.

*b. Pulmonary perfusion.*<sup>2</sup> Ligation of ductus arteriosus; afferent cannula in pulmonary artery, delivering the perfusion fluid under constant pressure and temperature; escape of perfusion fluid from open

<sup>1</sup> W. H. Manwaring, *Zeitschr. f. Immunitätsf.*, 1910, **8**, 2. The dogs giving this reaction were adult street dogs, sensitized by a single subcutaneous injection with 5 cc. to 25 cc. horse serum, kept in out-door kennels, at winter temperature (London), and tested at the end of from fifteen to thirty days. It is, of course, possible that other factors may be operative in more highly sensitized young dogs or puppies.

<sup>2</sup> W. H. Manwaring and Yoshio Kusama, *Jour. of Immunol.*, 1917, **2**, 157. Gives sketch of perfusion apparatus.

left auricle; cannula in trachea. During the perfusion, the lungs are alternately expanded and allowed to contract by air forced into the tracheal cannula, in a manner simulating the normal respiratory movements. Note is made of the resulting changes in resistance to inflation, and in the promptness and completeness of the expiratory collapse.

*Record.* In recording the observations, the following symbols are used:

(a) Lungs, liver:

N = Normal lungs or liver.

A = Anaphylactic lungs or liver ( $A_{14}$  = 14-day anaphylactic organ,  $A_{28}$  = 28-day anaphylactic organ, etc.)

(b) Perfusion fluid:

Lck = Locke's solution.

NB = Defibrinated normal guinea-pig blood (50 per cent NB = 50 per cent blood in Locke's solution, etc.)

AB = Anaphylactic blood ( $AB_{14}$  = 14-day anaphylactic blood, etc.)

GS = Goat serum (foreign protein).

(c) Pulmonary reaction, (i) *Alveolar fixation*:

0 = No change in pulmonary elasticity; lungs readily inflated, fully collapsible on expiration.

0 = Increased pulmonary flaccidity; tissues have a wilted appearance, edges of lobes curl over on expiration.

1 = Beginning alveolar fixation; lungs show a slight resistance to inflation, remain slightly expanded on expiration.

2 = Distinct alveolar fixation; lungs show a distinct resistance to inflation, remain half expanded on expiration.

3 = Marked alveolar fixation; lungs inflated with difficulty, remain three-quarters expanded on expiration.

4 = Total alveolar fixation; lungs of liver-like consistency, can neither be expanded nor collapsed by changes in air pressure in the tracheal cannula.

(ii) *Vascular resistance*:

0 = No change in rate of flow of perfusion fluid.

$\pm$  = Diminished rate of flow.

+

 = Cessation of flow.



(iii) *Edema*:

0 = No edema of lungs.

± = Slight edema, slight frothy fluid escapes from tracheal cannula.

+ = Marked edema.

Typical reactions of isolated normal and anaphylactic lungs, thus recorded, are shown in table 1.

TABLE 1  
*Typical pulmonary reactions*

PERFUSION FLUID	LUNGS	ALVEOLAR FIXATION										VASCU- LAR	EDEMA
		1 min.		2 min.		3 min.		4 min.		5 min.			
50 per cent NB + 0.25 per cent GS.....	N	0	0	0	0	0	0	0	0	0	0	0	0
50 per cent NB + 0.25 per cent GS. . . . .	A <sub>14</sub>	0	1	2	3	4	4	4	4	4	4	0	0

## A. PERFUSION OF NORMAL LIVERS

1. *Blood-free perfusion*

If 0.25 per cent to 1 per cent goat serum in Locke's solution is repeatedly perfused through a normal liver, no change in the toxicity of the perfusion fluid is observed on subsequent tests with anaphylactic lungs (table 2).

TABLE 2  
*Perfusion of normal liver: (1) Blood-free*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION										VASCULAR	EDEMA
			1 min.		2 min.		3 min.		4 min.		5 min.			
Locke + 0.5 per cent GS...	—	A <sub>15</sub>	0	1	2	4	4	4	4	4	4	4	0	0
Locke + 0.5 per cent GS...	N	A <sub>15</sub>	0	1	2	4	4	4	4	4	4	4	0	0

2. *Normal blood perfusion*

If 0.25 per cent to 1 per cent goat serum in 50 per cent defibrinated normal blood is repeatedly perfused through a normal

liver, a very slight reduction in the toxicity of the perfusion fluid is usually observed on subsequent tests with anaphylactic lungs. The change in toxicity, however, is never sufficient more than slightly to delay the anaphylactic response in these lungs (table 3).

TABLE 3  
*Perfusion of normal liver: (2) Normal blood*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION								VASCULAR	EDEMA		
			1 min.		2 min.		3 min.		4 min.				5 min.	
50 per cent NB + 0.5 per cent GS.....	—	A <sub>19</sub>	0	1	2	4	4	4	4	4	4	4	±	0
50 per cent NB + 0.5 per cent GS.....	N	A <sub>19</sub>	0	0	1	2	3	4	4	4	4	4	0	0

### 3. Anaphylactic blood perfusion

If 0.25 per cent to 1 per cent goat serum in 50 per cent defibrinated anaphylactic blood is repeatedly perfused through a normal liver, a complete loss of the passive anaphylactic response<sup>3</sup> is usually observed on subsequent tests with normal lungs (table 4).

TABLE 4  
*Perfusion of normal liver: (3) Anaphylactic blood: (a) Normal lung test*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION										VASCULAR	EDEMA
			1 min.		2 min.		3 min.		4 min.		5 min.			
50 per cent AB <sub>14</sub> + 0.5 per cent GS.....	—	N	0	0	0	1	1	2	2	3	3	4	0	0
50 per cent AB <sub>14</sub> + 0.5 per cent GS.....	N	N	0	0	0	0	0	0	0	0	0	0	0	0

The perfusion, however, gives no reduction in the toxicity on subsequent tests with anaphylactic lungs (table 5). It may give even a slight increase in this toxicity.

<sup>3</sup> W. H. Manwaring and Yoshio Kusama, Jour. Immunol., 1917, 2, 157. Gives limits of this response.

TABLE 5

*Perfusion of normal liver: (3) Anaphylactic blood: (b) Anaphylactic lung test*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION								VASCULAR	EDEMA		
			1 min.		2 min.		3 min.		4 min.				5 min.	
50 per cent AB <sub>13</sub> + 0.25 per cent GS.....	—	A <sub>13</sub>	0	?	1	2	3	4	4	4	4	4	±	0
50 per cent AB <sub>13</sub> + 0.25 per cent GS.....	N	A <sub>13</sub>	0	0	1	3	4	4	4	4	4	4	0	0

*Conclusions*

*Normal liver has a slight detoxicating action on foreign-protein-blood-mixtures, sufficient to prevent the passive anaphylactic response in subsequent tests with normal lungs.*

*The detoxicating action, however, is never sufficient to cause an appreciable difference in subsequent tests with anaphylactic lungs.*

## B. PERFUSION OF ANAPHYLACTIC LIVERS

*1. Blood-free perfusion*

If 0.25 per cent to 1 per cent goat serum in Locke's solution is repeatedly perfused through an anaphylactic liver, a very slight reduction in the toxicity of the perfusion fluid is usually observed on subsequent tests with anaphylactic lungs. The change in toxicity, however, is never sufficient more than slightly to delay the anaphylactic response in these lungs (table 6).

TABLE 6

*Perfusion of anaphylactic liver: (1) Blood-free*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION										VASCULAR	EDEMA
			1 min.		2 min.		3 min.		4 min.		5 min.			
Locke + 0.25 per cent GS..	—	A <sub>19</sub>	0	0	1	2	3	4	4	4	4	4	0	0
Locke + 0.25 per cent GS..	A <sub>19</sub>	A <sub>19</sub>	0	0	0	?	1	3	4	4	4	4	0	0

*2. Normal blood perfusion*

If 0.25 per cent to 1 per cent goat serum in 50 per cent defibrinated normal blood, is repeatedly perfused through an anaphylactic liver, a distinct reduction in the toxicity of the per-

fusion fluid is always observed on subsequent tests with anaphylactic lungs. The reduction in toxicity is at times sufficient to prevent the fatal anaphylactic response in these lungs (table 7).

TABLE 7  
*Perfusion of anaphylactic liver: (2) Normal blood*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION										VASCULAR	EDEMA
			1 min.		2 min.		3 min.		4 min.		5 min.			
50 per cent NB + 0.25 per cent GS .....	—	A <sub>17</sub>	0	1	2	3	3	4	4	4	4	4	0	0
50 per cent NB + 0.25 per cent GS .....	A <sub>35</sub>	A <sub>17</sub>	0	0	0	0	0	?	1	2	3	3	0	0

### 3. Anaphylactic blood perfusion

If 0.25 per cent to 1 per cent goat serum in 50 per cent defibrinated anaphylactic blood is repeatedly perfused through an anaphylactic liver, a marked reduction in the toxicity of the perfusion fluid is always observed on subsequent tests with anaphylactic lungs. The reduction in toxicity is usually sufficient to prevent all but a trace of the anaphylactic response in these lungs (table 8).

TABLE 8  
*Perfusion of anaphylactic liver: (3) Anaphylactic blood*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION										VASCULAR	EDEMA
			1 min.		2 min.		3 min.		4 min.		5 min.			
50 per cent AB <sub>14</sub> + 0.25 per cent Cs	—	A <sub>14</sub>	0	1	2	3	4	4	4	4	4	4	±	0
50 per cent AB <sub>14</sub> + 0.25 per cent Cs	A <sub>34</sub>	A <sub>14</sub>	0	0	0	0	0	0	0	0	?	1	0	0

### Conclusions

*Anaphylactic liver has a marked detoxicating action on foreign-protein-blood-mixtures, usually sufficient to prevent all but a trace of the anaphylactic response in subsequent tests with anaphylactic lungs.*

*The detoxicating action is more pronounced in perfusions with anaphylactic blood than with normal blood.*

## C. REACTION ANALYSED

## 1. Cellular factors

Parallel perfusions of normal and anaphylactic livers with duplicate foreign-protein-anaphylactic-blood-mixtures (table 9) show that the detoxicating action of the anaphylactic liver is not due solely to the presence of anaphylactic humoral elements. There is evidently a specific functional action of the sensitized liver cells.

TABLE 9  
*Cellular factors*

PERFUSION FLUID	LIVER	LUNGS	ALVEOLAR FIXATION								VASCULAR	EDEMA		
			1 min.		2 min.		3 min.		4 min.				5 min.	
50 per cent AB <sub>15</sub> + 0.25 per cent GS.....	N	A <sub>15</sub>	0	1	3	4	4	4	4	4	4	4	0	0
50 per cent AB <sub>15</sub> + 0.25 per cent GS.....	A <sub>40</sub>	A <sub>15</sub>	0	0	0	0	0	0	0	0	0	?	0	0

## 2. Quantitative changes in perfusion fluid

Parallel precipitin titrations<sup>4</sup> of normal and anaphylactic blood mixtures, before and after repeated passage through the anaphylactic liver, show no demonstrable decrease in the amount of the foreign protein, as a result of the perfusion (table 10).

TABLE 10  
*Quantitative changes in perfusion fluid*

Decreasing amounts of the perfusion fluid, centrifuged free from blood corpuscles, added to a constant amount of specific precipitin. + + + +, + + +, + +, + = precipitate; t = turbidity

PERFUSION FLUID	LIVER	PRECIPITIN TITRATION											
		2	4	8	16	32	64	128	256	512	1024	2048	
50 per cent AB <sub>35</sub> + 0.25 per cent GS.....	—	++++	+++	+++	+++	++	+	+	t	t	0	0	
50 per cent AB <sub>35</sub> + 0.25 per cent GS.....	A <sub>35</sub>	++++	+++	+++	+++	++	+	+	t	t	0	0	

<sup>4</sup> W. H. Manwaring, Yoshio Kusama and Harold E. Crowe, Jour. Immunol., 1917, 2, 511. Gives details of technique.

### 3. *Vaso-dilator and broncho-dilator substances*

Parallel tests of the perfusion mixture, before and after repeated passage through the anaphylactic liver, sometimes show a decrease or loss of the vaso-constrictor action usually present in the unperfused mixture (table 8).

The tests invariably show an acquired power of the perfusion fluid to cause a marked flaccidity or loss of tone of the pulmonary tissues (tables 7, 8, 9).

#### *Conclusions*

*The detoxicating action of the anaphylactic liver is not due solely to the presence of anaphylactic humoral elements. There is evidently an acquired detoxicating function of the fixed liver cells.*

*The detoxicating action is not due to a removal or destruction of the foreign protein in the perfusion fluid.*

*Evidence points to the explosive formation or liberation of vaso-dilator and broncho-dilator substances by the sensitized liver cells.*

#### SUMMARY

The liver of a normal guinea-pig, repeatedly perfused with a mixture of foreign protein and defibrinated normal or anaphylactic blood, produces little or no change in the toxicity of the perfusion mixture on subsequent tests with isolated anaphylactic lungs.

The liver of an anaphylactic guinea-pig, similarly perfused, usually renders the perfusion fluid almost completely non-toxic for these lungs.

This reduction in toxicity is not due to the presence of anaphylactic humoral elements, but to a specific functional action of the fixed hepatic cells.

The reduction in toxicity is not due to a removal or destruction of the foreign protein in the perfusion fluid.

Evidence points to the explosive formation or liberation of vaso-dilator and broncho-dilator substances by the sensitized liver cells.

## STUDIES IN ANAPHYLAXIS

### XXI. ANAPHYLAXIS IN DOGS. A STUDY OF THE LIVER IN SHOCK AND IN PEPTONE POISONING

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#### I. INTRODUCTION

The earliest studies on anaphylaxis, which were reported by Richet, were conducted on dogs. It was only after the lapse of several years that the guinea-pig, and, somewhat later, the rabbit, were found to be susceptible to anaphylactic shock. The shock of the dog has been found to differ in certain important particulars from that of the guinea-pig. Under typical conditions, the death of the latter is either immediate, or a matter of minutes, while that of the former usually occurs only after the lapse of hours. In the dog, the cardio-vascular mechanism is an important factor, and the tremendous fall in blood pressure is probably the direct cause of death. In the guinea-pig, this vasomotor disturbance plays no part in acute shock, but in its stead there occurs a spasmodic closure of the bronchioles by their circular musculature, with inspiratory fixation of the lungs, and asphyxia. These striking differences in the manifestations of anaphylactic shock in the two species have naturally influenced the theory of the respective mechanisms involved. Thus Doerr (1) is willing to grant that shock in the guinea-pig is simply the result of a reaction between cellular antibody and circulating antigen; as regards the dog, on the other hand, he inclines to the belief that protein decomposition, with the formation of intermediate toxic substances, is chiefly responsible for the production of the symptoms. This latter view, originally pro-

pounded by Biedl and Kraus, has received important support through the work of Jobling (2), who found that the blood of dogs in anaphylactic shock, constantly shows an increase in the products of protein disintegration. To these products Jobling ascribed toxic properties, and considered them to be the immediate cause of the symptoms.

It was the first object of the present study to determine the significance of these changes in the blood. Dogs at the height of anaphylactic shock were exsanguinated, and this blood was then injected in large amounts into normal animals, in order to discover whether such blood is possessed of toxic properties. As a matter of fact, the transfused dogs failed to show any ill effect from the anaphylactic blood. It seems fair to conclude, therefore, that the symptoms of the anaphylactic dog cannot be attributed to the presence of toxic substances in the blood. On the other hand, even though without influence upon the development of shock, the changes in the blood are in themselves of considerable interest. The mechanism which results in these changes had not as yet been determined, and therefore became a further subject of investigation. A comparison of the blood changes which occur in phosphorus and in chloroform poisoning reveals a striking similarity to those of anaphylaxis. Inasmuch as phosphorus and chloroform act as hepatic poisons, it is generally believed that the degenerative processes which they set up in the liver are responsible for the alteration in the blood. If this analogy be applied to anaphylaxis, it would seem to indicate that in the latter condition, likewise, the liver is the seat of important changes. The further pursuit of this notion led to the discovery of additional data, both experimental and pathological, which support the theory that the entire syndrome of canine anaphylaxis is due to the reaction of the liver.

## II. TRANSFUSIÓN EXPERIMENTS

### *Technique*

The first step in the experiment was to put dogs into a condition of severe anaphylactic shock. It is well known that this



can be accomplished with considerable regularity by giving an intravenous injection of antigen two or three weeks after the sensitizing dose. The technique hitherto employed has the disadvantage, judged from the standpoint of the requirements of the present study, that the dogs do not die until after several hours. If, therefore, one wishes to take blood from the animals in a condition of extreme shock, just previous to the fatal issue, it becomes necessary to observe them over many hours, often even over night. In order to obviate this difficulty, a procedure has been devised<sup>1</sup> which almost regularly produces anaphylactic death within a period of one half hour to one hour. The dogs are prepared by two injections of 5 cc. of horse serum, the first of which is given subcutaneously, and the second preferably intravenously. After an interval of two or three weeks or more, an intravenous injection of 20 cc. of horse serum is given. This amount, though considerably larger than that employed by most students of anaphylaxis in dogs, is still very far below the toxic dose for unsensitized animals. Thus normal dogs weighing between 10 and 15 pounds have received as much as 50 cc. of horse serum intravenously without showing any effects, while 20 cc. proves rapidly fatal to sensitized dogs weighing over 40 pounds. The symptoms following upon this second injection are characteristic and almost constant. The dog immediately vomits or retches, and generally has a number of evacuations of the bowels. Within five minutes it begins to stagger and to drag its hind legs. Following this preliminary stage comes a period of severe collapse, which, as a rule, appears within ten minutes of the injection. The animal lies on its side and does not respond to any stimulation. Some animals show at first either a fine tremor of the muscles of the extremities, or a coarse clonus composed of short excursions. These soon cease, and the animal is practically immobile, except for the respiratory movements. Respiration is either shallow and rapid, or labored, and gives the impression of marked dyspnoea. During this stage, which terminates, usually within thirty minutes, with the death of the

<sup>1</sup> A similar method was employed by Auer, and is described in Forcheimer and Billings "Therapeutics of Internal Diseases." Appleton's, 1914, 5, 88.

animal, the other characteristic features of anaphylaxis make their appearance. The blood pressure sinks so low that the carotid pulse can scarcely be detected. If blood is aspirated from the veins, it is found to have lost its coagulability to such an extent that it remains fluid for several days. In order to secure the maximum amount of blood, very large dogs, weighing from 25 to 60 pounds, were selected for the experiment whenever available.

This rapidly fatal form of shock differs, as has been said, from the type generally hitherto described. Richet (3) states that "Death in less than two hours is extremely rare in the dog." The pathological features of this type of shock also differ in certain important particulars, as will be shown in another section of this paper.

The second step in the experiment consists in obtaining blood from the anaphylactic animals. When it has become apparent from the general symptoms that death is imminent, the dog is laid on the table, and is bled from the exposed carotid. At this stage the animal is practically unconscious and insensitive to pain; nevertheless the skin should be anesthetized with carbolic ointment. Owing to the extremely low pressure, the blood runs very slowly, and often merely trickles from the vessel. With the death of the animal during this procedure, no further blood is obtainable from this source. The abdomen is now opened by a median incision, which is prolonged up to the neck, and the thorax is opened widely by bilateral section of the ribs. A needle attached to an aspirating syringe is plunged into the large accessible veins, notably the cavae and vena portae, all of which are generally found engorged with fluid blood. By means of this procedure the yield of blood from the carotid can be materially increased. The blood is incoagulable and remains fluid for many hours, or even days. In many of the experiments, nevertheless, sodium citrate was added in amounts ranging from 0.1 to 0.5 per cent of the blood. Whenever this was done, an uncitrated sample of the same blood was put aside in order to establish its incoagulability, inasmuch as this feature furnished an important criterion of the depth of shock

at the time of bleeding; in no instance throughout the entire series did this test sample coagulate within a period of twenty-four hours. Thus the character of the blood itself gave a guarantee that it was taken in a condition of very profound anaphylactic shock. In two of the experiments the animal was allowed to die without any interference, and the blood was then aspirated from the veins post mortem. With the latter procedure, however, considerably less blood is obtainable. The physiological effect of such post mortem blood upon normal dogs does not differ from that of blood taken in severe shock during life. In some of the experiments the blood was immediately placed in an incubator and kept there for the few minutes consumed in the preparation of the second dog, in others, it was kept in the ice box.

The third step of the experiment consists in the introduction of the anaphylactic blood into normal dogs. For this purpose it is advisable to keep the animal under light anesthesia. Parenthetically it should be added that ether anesthesia does not in the least inhibit acute anaphylactic shock with death in dogs. Small dogs preferably were utilized, in order that their blood volume might be replaced as completely as possible by the anaphylactic blood. For the transfusion itself various procedures were tested. The Unger method, which consists in connecting the circulation of the two dogs by means of a syringe with a two way stopcock, was successfully employed in one experiment. It was found, however, that the conditions of the experiment could best be served by a very simple technique, and the following method was eventually adopted in practically all the experiments. The external jugular and the common carotid were both exposed, and were ligated distally. A large caliber needle attached to rubber tubing controlled by a clamp was then inserted into each of these vessels. At alternate intervals blood was withdrawn from the carotid, and the anaphylactic blood was allowed to run into the vein from a glass funnel connected by rubber tubing with the intrajugular needle. Certain necessary precautions of technique, such as the prevention of air bubbles, the securing of the needles in situ, and so forth, need not be speci-

fied in detail. By this method it is possible to replace a very large proportion of the animal's own blood with anaphylactic blood. When the procedure is completed the needles are withdrawn and the vessels are ligated proximally. The skin incisions are sutured, the ether stopped, and the animal is removed from the operating board.

Up to the present time, ten such transfusions have been performed on normal dogs. The amount of anaphylactic blood transfused in each experiment has ranged between 100 and 600 cc., the average being 312 cc. The following table presents the important data concerning the dogs transfused in these experiments.

NUMBER OF DOG	WEIGHT IN POUNDS	VOLUME OF BLOOD BY CALCULATION	AMOUNT OF BLOOD TAKEN	AMOUNT OF ANAPHYLACTIC BLOOD TRANSFUSED
1	15½	530 cc.	150 cc.	200 cc.
2	20	680	80	100
3	18	620	?	130
4	11	375	100	125
5	12	410	250	320
6	11½	395	300	340
7	26	890	300	300
8	11	375	335	600
9	17½	600	400	450
10	12	410	500	550

It is evident that in some of the animals the transfusion represented only a moderate proportion of the animal's blood. In certain of the experiments, however, particularly numbers 5, 6, 8, 9 and 10, the replacement was more nearly complete. An animal weighing from 11 to 12 pounds should have approximately between 375 and 400 cc. of blood; upon this calculation it will be seen how considerable a portion of anaphylactic blood was in circulation in the normal animal at the end of the operation. As a matter of fact, in three of these five experiments, in which blood was taken from the jugular between ten and twenty minutes after the transfusion was over, it was found to be almost incoagulable, thus affording convincing proof of the degree to which the normal blood had been substituted.

The outcome of all these experiments was identical. Within about ten minutes the animals "came out" of the ether, as is usual following experiments of this duration, and were able to walk about almost normally. Throughout this period they acted exactly as though nothing further had been done to them than to keep them very lightly under ether for a period of one-half to one hour. There were no symptoms of any kind, resembling anaphylaxis. The further observation of these animals for periods of several days or weeks failed to reveal any peculiarities in their behavior, or any disturbances of health. Appetite was good, all functions normal, and the weight little, if at all, altered.

Some of the animals were killed at intervals of several hours to days after the operation, and were carefully autopsied. None of them showed any of the post mortem changes which are so strikingly characteristic of anaphylaxis. Thus it may be said that the experiments fail to give any support to the theory that the blood of anaphylactic animals contains toxic substances, or produces any harmful effects whatever upon the organism.

The following detailed protocol of three of the experiments are added in order to illustrate the method of procedure and the results obtained.

*Experiment 8.* Dog 28, December 4, weight 39 pounds.

December 4. Received 5 cc. of horse serum subcutaneously.

December 7. Same treatment.

December 27. Weight, 43 pounds. At 11.50 placed on operating board; 10 cc. of blood aspirated from vein and found to coagulate in five minutes.

11.55. 20 cc. of horse serum injected intravenously. The dog was immediately removed from the board and at once began to vomit and to strain.

12.05. Dog lying on side, weak, labored breathing.

12.10. Dog in collapse, carotid pulse weak. Dog placed on board and carotid exposed.

12.18. Bleeding begun; arterial pressure very low; 10 cc. of blood reserved to determine coagulability, the remainder made up to 0.5 per cent sodium citrate; 400 cc. thus obtained.

12.25. Dog dead; aspiration of veins begun; 200 cc. obtained and citrated.

12.50. Aspiration finished; 600 cc. obtained in all. The sample of uncitrated blood showed no coagulation two days later.

Post mortem examination showed the typical congestion of the liver.

The blood thus obtained was introduced into dog 21 immediately, as follows:

Dog. 21, normal. December 27, weight 11 pounds. Placed on board at 1 o'clock; needles inserted into the carotid artery and the jugular vein. Blood was taken from the carotid and introduced into the jugular, alternately. The first drawing represented about two-fifths of the estimated amount of blood in circulation.

TIME	AMOUNT INTRODUCED	AMOUNT DRAWN
1.14 (a)		150
1.15	135	
1.22		50
1.23	60	
1.26		75
1.27	75	
1.30 (b)		100
1.32	100	
1.37 (c)		100
1.39	130	
1.45		60
1.48	100	
Totals.....	600 cc.	535 cc.

The wounds were sutured.

1.56. Dog off table.

2.05. Dog sitting up but unable to walk steadily; carotid pulse good.

2.06. Mild chill.

2.20. Apparently almost normal; walking about.

At a, b and c, samples of blood kept, to determine coagulability; a clotted in seven minutes, b and c were still fluid at 4.45.

The subsequent course of this dog gave no evidence of ill effects from the transfusion. On the next day the animal ate, walked and played as usual. On January 2, no symptoms of any kind having

developed, the animal was killed. The organs in the gross seemed normal, and the microscopic sections revealed no abnormalities.

*Experiment 9.* Dog XXX; December 12; weight 59 pounds.

December 12. Received 5 cc. of horse serum subcutaneously.

December 14. 5 cc. of horse serum intravenously.

January 19. 11.00 a.m. 30 cc. of horse serum injected intravenously. Off board.

11.05. Vomiting.

11.10. Diarrhea; severe prostration; dyspnoea.

11.20. Dog placed on operating board and carotid exposed. Pulse very poor; 900 cc. of blood drawn from carotid and kept without the addition of citrate.

11.35. Aspiration of 150 cc. from veins.

The post mortem examination showed intense congestion of liver.

A sample of the blood taken from the carotid of this animal was kept, and failed to show any evidence of clotting within three days.

The blood thus obtained was introduced into dogs 25 and 26. Dog 25, normal, January 19, weight  $17\frac{1}{2}$  pounds. Prepared in the usual fashion. Blood drawn and injected alternately as follows:

TIME	AMOUNT INTRODUCED	AMOUNT DRAWN
1.03		200
1.05	250	
1.12		100
1.17	100	
1.19		100
1.22	100	
Totals.....	450 cc.	400 cc.

1.30. Wounds sutured and dog taken off table. Pulse normal.

1.45. Dog walking about. Pulse normal.

January 20, dog perfectly normal. Blood aspirated from the jugular clotted in 5 minutes.

*Experiment 10.* Blood used from dog 30, obtained as described in experiment 9, injected into dog 26. Dog 26, normal. January 19, weight 12 pounds. Prepared in the usual fashion. Blood drawn and injected alternately as follows. The first drawing represented almost one half of the estimated amount of the animal's blood.

TIME	AMOUNT INTRODUCED	AMOUNT DRAWN
12.13	275	180
12.16		
12.24		120
12.26	50	
12.27		100
12.29	100	
12.31 (a)		100
12.33	125	
Totals.....	550 cc.	500 cc.

Blood drawn at (a) was kept for observation and found to be incoagulable.

12.40. Dog taken from the table. Pulse normal.

12.50. Dog standing up and had a slight chill; carotid pulse strong.

1.00. Dog walking about.

1.15. Dog apparently quite normal.

On the following day blood aspirated from the jugular was found to clot in three minutes.

### III. THE INCOAGULABILITY OF THE BLOOD

The changes which occur in the blood of the anaphylactic dog are of great interest in themselves, even though they do not appear to contribute to the production of the symptoms. These changes involve both the coagulability and the chemical composition of the blood. (The latter will be considered in connection with peptone shock.) In 1909 Biedl and Kraus pointed out the fact that the coagulability of the blood is markedly diminished during anaphylactic shock in the dog. Coagulation may be only delayed, or may be completely abolished. If a clot forms, it is less firm and tenacious than normally, and does not contract as energetically. A similar change, though slighter in degree, characterizes the blood of rabbits in anaphylaxis, and of guinea pigs in case the shock is protracted.

Although no experimental studies have as yet been made, theoretical considerations point to the liver as probably chiefly concerned in the production of this latter change in the blood. In



order to secure direct evidence upon this problem, the following experiments were devised. The object in mind was to perfuse the isolated liver of a sensitized dog with the antigenic substance, in order to determine whether under these circumstances the organ produces an anti-coagulant. Obviously, the simplest method of attaining this object is to remove the liver from the body, and to perfuse it by means of a cannula in the portal vein, the process being conducted in a warm chamber. It is possible to perform this experiment with blood as the perfusing medium in case an anti-coagulant is added. Using citrate as an anti-coagulant, it was found necessary to make the blood up to 1 per cent of sodium citrate, inasmuch as weaker mixtures clot spontaneously in the liver, in spite of the fact that 0.25 per cent suffices to keep the blood fluid in flasks. After the perfusion is finished, the citrate is neutralized by the addition of the requisite amount of calcium chloride in solution, and the coagulation time is determined. As a matter of fact, the experiment as above described was carried out a number of times, but it was regularly found that the blood had become even more rapidly coagulable. Nevertheless, the conditions are so artificial that this negative result permits of no definite conclusion. The presence of the citrate constitutes a disturbing factor, since it has been shown that citrate alone, when injected intravenously, markedly increases the coagulability of the blood.

The same type of experiment may be conducted under more natural conditions, according to a procedure devised by Doyon for a different purpose. The sensitized dog is bled almost to death from the carotid. By a medium abdominal incision and by removal of the sternum and adjoining cartilages, the organs are then exposed. A cannula is placed in the vena portae, the inferior cava is ligated just below the liver, and a second cannula is placed in the inferior cava above the diaphragm. The cannula in the vena portae is connected by means of a short tubing with the carotid of a normal dog, and thus a blood circulation is maintained through the organ. At a chosen time the antigen is injected into the connecting tubing. Blood is collected at intervals from the superior cava, and its coagulation time is deter-

mined. The results of this experiment, when satisfactorily carried out, afford ample demonstration of the fact that the reaction of the sensitized liver distinctly lowers, or abolishes, the coagulability of the blood. It seems probable, therefore, that this change in the blood is simply the resultant of a cellular reaction of the sensitized liver. The following experiments illustrate the method of procedure.

*Experiment 1. Dog.*

October 5. 5 cc. horse serum subcutaneously.

October 8. 5 cc. horse serum intravenously.

October 30. Weight 50 pounds. Doyon procedure.

11.40. Carotid-portal circulation established.

11.45. Cava blood coagulates in three minutes.

11.46. 10 cc. 20 per cent horse serum in normal salt, injected into tubing.

11.50. Coagulation time, two minutes.

12.00. Coagulation time, thirteen minutes.

Blood clotted in tubing and in vena portae.

Experiment discontinued.

*Experiment 2. Dog.*

Same preliminary treatment as in previous experiment.

October 29. Doyon method.

12.20. Carotid-portal circulation established.

12.23. Cava blood, clotting time two minutes.

12.25. 3 cc. of horse serum into tubing.

12.34. 3 cc. of horse serum into tubing.

12.53. Cava blood remains unclotted after twenty-four hours.

When normal livers were perfused according to this method, the coagulation time of the blood in the cava was either unchanged, or accelerated, after the addition of horse serum. The result of this experiment is conclusive, up to a certain point. It demonstrates the participation of the liver, but it does not necessarily exclude the other organs from a share in the process.

#### IV. THE VASOMOTOR DEPRESSION

A striking and characteristic feature of anaphylactic shock in dogs is the fall of blood pressure. It occurs almost immedi-

ately upon the injection of the antigenic substance, and persists until death; if the animal recovers from the shock, the pressure gradually rises. In the opinion of most students of the subject, the fall in pressure constitutes the cause of death.

Although much is still in doubt, certain factors in the fall of pressure have been established. Manwaring's (4) experiments, supported by those of Voegtlin and Bernheim (5) and of Deneke (6) render it certain that the liver is essential to the vasomotor depression, inasmuch as the latter fails to supervene if the liver is excluded from the circulation. Furthermore, it is quite generally understood that the post mortem appearances correspond in general with those of surgical shock, inasmuch as there is an accumulation of blood in the large veins of the abdomen. Pearce (7) says: "It is essentially the condition described as bleeding into the veins of the abdomen."

The intermediary processes which lead to this result have not been entirely cleared up. Biedl and Kraus (8) elaborated a theory, which seemed to offer a satisfactory explanation and has been fairly generally accepted. They believe that the injection of antigen into the sensitized animal gives rise to the production of peptone-like bodies, and that the latter induce vasomotor depression by paralyzing the vasoconstrictor nerve endings in the vessels. In support of this view they state that adrenalin, which acts upon the vasomotor terminals, fails to raise the pressure to any extent when injected during anaphylactic shock, whereas barium chloride, which stimulates the vascular musculature directly, is very effective. On the other hand, a number of their illustrative curves, notably those on Plate VII, demonstrate a sharp and very pronounced effect from adrenalin given in peptone shock, while the authors declare that "the effects of peptone are identical in every particular and in the minutest detail with those of an anaphylactic injection." However, these authors conclude that the fall in pressure is due to a paralysis of the nerve terminals in the vessels. Pearce and Eisenbrey (9) repeating these experiments, report that "while barium causes an increase in pressure, adrenalin does likewise; the latter, however, is but slight." They therefore modify Biedl and Kraus' expla-

nation and interpret their results as pointing to "an influence on the nerve endings" rather than a paralysis. Recent studies by Simonds (10) have brought out the additional fact that the injection of nicotin during anaphylactic shock produces a rise which is even more striking than under normal conditions. Now nicotin is known to act upon the blood vessels through the vasoconstrictor mechanism, so that this result is quite incompatible with the view that the terminals are paralyzed or markedly depressed. Simonds suggests that the nicotin may produce its effects by stimulating respiration and thus causing the aspiration of blood from the abdomen into the thorax; this novel hypothesis, however, is quite unsupported by confirmatory experiments. Thus it is evident that the pharmacological analysis of anaphylactic shock has not led to concordant results, and that the actual mechanism of the abdominal congestion does not appear to be definitely settled.

As an alternative hypothesis, I have suggested (11) that the engorgement of the liver results from the local reaction of the sensitized hepatic cells to the antigenic substance. This view was prompted by the observation that in animals dying acutely of anaphylactic shock within an hour after the injection of the antigen, the liver is practically the only organ of the abdomen which is congested, while the stomach and intestines may be even less well supplied with blood than under normal conditions. The picture is, therefore, not that of a splanchnic paralysis, but rather of isolated hepatic congestion. If an animal lives for a number of hours, however, the gastrointestinal tract may become the seat of severe congestion, which is therefore probably a secondary result of the blockage in the liver.

The following experiment showed that congestion is, in fact, a direct and immediate effect of the response of the sensitized cells of the liver to the antigen.

*Experiment.* A dog was sensitized by the intravenous injection of 5 cc. of horse serum. After an interval of three weeks, the animal was put under ether and the abdomen opened by a median epigastric incision. The surface of the liver was punctured by a very fine needle attached to a hypodermic syringe, and a drop of a 10 per cent solu-

tion of horse serum was injected just below the surface. Almost immediately the point of injection became the site of an intense and sharply localized congestion, measuring perhaps  $\frac{1}{2}$  inch in diameter, and over this area the surface of the liver was distinctly raised. The duration of this reaction has not been accurately determined, but it has been seen to persist for more than one-half hour.

This experiment has repeatedly been performed on sensitized animals, and always with the identical result. On the other hand, normal dogs generally fail to show the slightest effect when the liver is injected in this manner; at most there is a very slight, purely traumatic, reaction. It is therefore clear that the response must be interpreted as an anaphylactic or allergic reaction. There is one feature of the experiment, however, which requires explanation. If the liver of a dog sensitized with horse serum be injected with rabbit serum, there also follows a congestive reaction. This might seem to indicate that the reaction is not specific, but represents simply a generalized irritability. In previous studies I have emphasized the fact that sensitization in guinea-pigs is often not sharply specific, but that animals previously injected with human serum, for example, may react to rabbit serum. In order to determine whether the crossed reaction of the liver represents a crossed anaphylactic response, a dog sensitized against horse serum was given an intravenous injection of rabbit serum. The animal immediately manifested unmistakable symptoms of shock, characterized by marked prostration, and the coagulation time also rose from four to ten minutes. This result indicates that the crossed congestion reaction is a true index of sensitization. If the liver of a dog sensitized to horse serum is injected with egg white, there is no reaction. But the liver of a dog previously injected with egg white may respond to an intrahepatic injection of egg white in the manner described, whereas it fails to react to horse serum. Dogs do not, however, regularly become sensitized to egg white, and the latter experiment succeeds only in a certain proportion of cases.

In order to throw further light on this question, the following experiment was devised.

*Experiment.*—A dog was sensitized by the intravenous injection of 5 cc. of horse serum. Two weeks later the dog was etherized and the liver exposed by a median epigastric incision. A subsurface injection of a minute amount of horse serum into the liver provoked an immediate congestive reaction. Into the right branch of the portal vein 0.5 cc. of horse serum was then injected. The right lobe of the liver at once became intensely congested, while the left lobe showed no change. Blood was aspirated from the jugular five minutes later and found to be incoagulable. After fifteen minutes, although etherization had been discontinued at the moment of portal injection, the animal appeared to be in shock, and the carotid pulsation was very small. The dog was killed and the organs examined. The large abdominal veins were distended, but the abdominal viscera were not congested, with the exception of the liver. The right half of the liver was deeply congested, cyanotic in color, and firm to pressure, and the cut surface bled freely. The left half was only slightly, if at all, congested.

This experiment shows that the congestive condition of the liver in anaphylaxis is due to local, and not to general conditions. Only that part of the liver which comes into direct contact with the antigen takes part in the reaction, while the adjoining areas seem to show merely a collateral injection. If the general vasomotor mechanism were responsible for the dilatation of the hepatic vessels, the entire organ would necessarily be involved, together with the remainder of the areas controlled by the splanchnic nerves. The fact that the congestive reaction is a local anaphylactic response does not, however, present a complete explanation of the phenomenon. It is not probable that the effect of the antigen is exerted directly upon the capillary endothelia of the hepatic vessels, inasmuch as a similar effect would probably be exerted upon all such endothelia; yet when the antigen is injected into the general circulation the other organs do not noticeably participate in the congestive reaction. It is probably correct to assume, therefore, that the sensitized hepatic cells respond in the first instance to the antigen, and that the congestion is secondary to the irritation of the parenchyma cells. It is noteworthy that the liver presents a marked circulatory disturbance immediately upon the injection of the antigen into the

portal, or one of its branches. It is difficult to detect a latent interval. The ensuing prostration comes on with great rapidity. This general effect, and also the altered coagulability of the blood, are induced by means of very much smaller amounts of antigen when the injection is given directly into the portal, than when it is given into the jugular. In the present instance, 0.5 cc. of horse serum gave symptoms of such severity as would have required an injection of perhaps 10 or 20 cc. into the general circulation.

The same experiment has been repeated a number of times, using varying amounts of horse serum, and always with the same result. If, however, larger amounts of horse serum are employed in the portal injection, the hepatic reaction becomes correspondingly more intense. If very large amounts are injected, as, for example, 5 cc., the entire liver may participate in the congestion, although the individual lobe corresponding to the portal branch injected always evidences an earlier and more pronounced involvement. The larger injection presumably permits of the passage of a sufficient amount into the general circulation to mediate a response of the remainder of the organ.

The explanation here offered depends upon the assumption that the congested liver may take up such an amount of blood as practically to deplete the general circulation. It is possible by means of calculation to arrive at an approximate estimate of both of the factors concerned in this assumption, namely the amount of blood in circulation and the amount taken up by the liver, and thus to test the assumption. The volume of blood in circulation is calculated as equivalent to one-thirteenth of the total body weight, this being the proportion indicated by recent text books of physiology. Of this total volume, according to Stuart (12), and other authors, the liver contains "rather more than one-fourth." In other words, the total volume of blood in circulation outside of the liver amounts to  $\frac{1}{13} \times \frac{3}{4}$ , or  $\frac{3}{52}$ , of the weight of the animal. The excess volume of blood in the congested liver may be ascertained by subtracting from the actual weight of this liver the calculated weight of the normal liver for an animal having the same weight; the difference must correspond to the

only possible variant, namely the blood content. The determination of the weight of the normal liver from the weight of the whole animal is not entirely a simple matter, inasmuch as, in all probability, there are variations depending upon such factors as muscular and skeletal development in the various breeds of dogs. Weighings were made of a number of animals in order to reach a basis for such a calculation, and the conclusion was reached that the liver weighed on average about one-twenty-seventh of the total body weight. The only data which could be discovered in the literature, in Welcker and Brandt's (13) tables, confirmed this finding, inasmuch as the weight of the liver is there given as 3.69 per cent of the total weight. In order to make conditions as nearly constant as possible, the animals were all kept on a constant diet, and were always used about twenty-four hours after the previous meal. Calculations made on the basis described disclosed the fact that the liver of dogs dying in anaphylactic shock always contains a large proportion of the circulating blood, varying from 30 to 60 per cent. The lowest figures were those obtained for the liver of animals which had been bled to death for the transfusion experiments previously described. Obviously such livers would no longer contain the full volume of blood. Where the animals were allowed to die in shock the figures were invariably very much higher. A typical example of the method of calculation and of the result is afforded by the following observation. The results are essentially the same, whether the antigen is injected into the portal or into the jugular vein.

*Experiment.* A dog weighing 9.7 kilos, received 2.5 cc. of horse serum into the portal vein. The liver immediately became cyanotic in color and turgid with blood, and within less than five minutes attained a size which was not exceeded during the remaining fifteen minutes of life. The intestines also gradually became slightly congested, and the abdominal veins swollen. The dog promptly passed into intense shock with all the characteristics of the anaphylactic reaction. Twenty minutes after injection the animal died, and the liver was immediately removed after clamping the inferior cava above and below the diaphragm, and the portal vein. In spite of these precautions, a considerable amount of blood escaped from the organ and



was lost, thus appreciably diminishing the final weight. The weight of the liver was found to be 700 grams. These two weights, 9.7 kilo for the whole animal, and 700 grams for the liver, afford the basis for the following calculations:

Total volume of blood in circulation: $9700 \times \frac{1}{13}$ .....	746 cc.
Volume of blood contained in normal liver: $746 \times \frac{1}{4}$ .....	186 cc.
Volume of blood in circulation outside of liver.....	560 cc.
Calculated weight of normal liver: $9700 \times \frac{1}{27}$ .....	356 grams
Ascertained excess of blood in liver $700 - 356$ .....	344 grams
Proportion of circulating blood taken up by congested liver: $\frac{344}{560}$ .....	61.5 per cent

In this experiment, therefore, it is found that 61.5 per cent of the blood available for the general circulation is contained in the liver. But these figures do not give the whole story. The congestion of the liver is so intense that circulation of the portal blood is very markedly interfered with, so that the intestines very shortly begin to show a moderate grade of congestion, and the abdominal veins become somewhat swollen. If we consider that there is very much less blood than normally in the general circulation, it is clear that the abdominal contents would be extremely anemic if they contained only their fair proportion of that blood. The congestion, though moderate, shows that obstruction to the portal circulation through the liver results in the accumulation of considerable amounts of blood in the abdomen, with still further depletion of the general circulation.

If it is realized that these conditions develop with very great rapidity, there seems no reason to question their adequacy in producing an immediate and extreme fall of pressure, with all of the consequent symptoms of shock, and rapid death. Lesser degrees of the same reaction would naturally lead to delayed shock, with the secondary results of the intestinal congestion, such as bloody diarrhea and hemorrhage into the wall of the bowel, as seen in the less acute types of hemorrhage.

## V. PATHOLOGY

It is not intended in this place to give the complete results of a study of the pathology of anaphylactic shock in dogs, but merely to indicate certain outstanding features.

The typical result of a post mortem examination of a dog dying in anaphylactic shock is portrayed by Richet as follows:

The pathological anatomy of animals that have died of acute anaphylaxis scarcely gives any positive information. There is intense congestion with interstitial haemorrhage in the whole gastro-intestinal tract. The lungs are congested and sometimes also the endocardium and pleura, lesions that can be accounted for by the intense vasoparalytic dilatation in all the viscera.

All authors have laid stress preëminently upon the intense, often hemorrhagic, congestion of the gastro-intestinal tract.

In dogs dying within one hour, the gross appearances do not at all correspond to Richet's description. The congestion of the intestines is slight, or moderate, in degree, and may be altogether absent. This finding corresponds to the comparative lightness of the gastro-intestinal symptoms (the vomiting and diarrhea) of this peracute form of shock. It suggests the possibility that the gastro-intestinal symptoms and changes may be late, or secondary, results. The pulmonary changes described by Richet are practically never in evidence. On the other hand, the condition of the liver dominates the pathological impression, and presents a picture such as is rarely, if ever, seen under any other circumstances. The organ is tremendously swollen. Its color is intensely cyanotic. Upon section, the cut surface bleeds freely. The pancreas, also, may show a slightly greater degree of congestion than is normal. A very moderate degree of congestion is sometimes seen in the kidneys, the suprarenals, and the spleen. Microscopic section of the organs reveals marked changes in the liver. There is, in the first place, an intense congestion. The parenchyma cells show various changes, which are not present in the same degree in all the animals. There is cloudy swelling in varying degree. The cells may be greatly vacuolated, and their boundaries may be very indistinct. One specimen showed a high degree of disseminated necrosis. The other organs present no definite microscopic alterations.

## VI. PEPTONE SHOCK

The preceding observations throw light upon the so-called peptone theory of anaphylactic shock. It is well known, as a result of experiments reported by de Waele and by Biedl and Kraus, that the injection of peptone intravenously in a dog produces a series of symptoms highly suggestive of anaphylactic shock. The blood becomes incoagulable, the arterial pressure falls to a very low figure, vomiting and diarrhoea occur, and the dog may then recover, or may die in shock. Some of these observations, as for example, the incoagulability of the blood, were recorded long before they were noted by Biedl and Kraus, but these authors were the first to indicate the far reaching analogy between peptone intoxication and anaphylactic shock. Biedl and Kraus inferred that in anaphylaxis the antigen-antibody reaction in vivo leads to protein degradation, with the formation of toxic peptone-like substances.

This theory of peptone shock as the cause of anaphylaxis has recently been modified and strengthened by Jobling. Biedl and Kraus believed that the peptone was derived by proteolysis from the antigen, whereas Jobling has maintained that it is derived from the animal's own plasma. In support of the peptone theory Jobling has adduced the fact that the blood of anaphylactic dogs actually shows an increase in the intermediate and end products of protein disintegration. This finding he interprets as indicating the "Cleavage of serum proteins (proteoses) through the peptone stage to aminoacids, and an intoxication by these peptones."

In so far as this peptone theory is based upon concrete evidence, such as the chemical alteration of the blood during shock, it is not supported by the previously described transfusion experiments, which demonstrate that these chemical changes are not associated with toxic properties. If, however, it is maintained that the toxicity of the blood depends upon the peptones alone, which rapidly break down to non-toxic products, the chemical data of themselves fail to establish this contention. Jobling was able to demonstrate an increase of incoagulable nitrogen in ana-

phylactic blood amounting to barely more than 5 mgm. in 100 cc. of serum. If we make the extreme assumption that all of this N existed at one time in the blood as peptone, there would be an increase of perhaps 35 mgm. of peptone at such a moment in 100 cc. of serum. On the other hand, it is necessary to inject about 500 mgm. of Witte's peptone to 75 cc. of whole blood (0.5 gram per kilo of body weight) in order to kill a dog. Thus, even on the basis of the preceding assumption, the chemical changes in anaphylactic blood represent less than one-fortieth of that required in order to sustain the analogy with peptone shock.

There is another phase of the subject of peptone shock which is of considerable interest, namely, its underlying mechanism. This has been successfully worked out for at least one of the characteristic features of that type of shock, namely the incoagulability of the blood. Peptone, when added to freshly drawn blood in vitro, has only a very slight effect on coagulation time (Schmidt-Mülheim (14)). Grosjean (15) showed that in order completely to inhibit coagulation in vitro, as much peptone is required as would correspond in vivo to 15 grams per kilo, whereas, actually, 0.15 to 0.3 gram per kilo is quite sufficient, when given intravenously, to render the blood entirely incoagulable. It is clear, therefore, that there must be an accessory mechanism of the body which tremendously reinforces the very slight anticoagulant action of peptone. Contejean (16) believed that this accessory mechanism is furnished by the reaction of the tissues in general, but chiefly of the liver. Subsequent studies have indicated that the liver is the exclusive site for the production of anticoagulants in response to the stimulation of peptone. Gley and Pachon (17) destroyed the liver cells by an injection of acetic acid into the ductus choledochus, and then found that the intravenous injection of peptone produced no change in coagulation time. The work of Gley and Pachon, Camus and Gley, Doyon, and Delezenne, all points to the same conclusion. Thus it appears that peptone has a marked effect upon the liver, and that the incoagulability of the blood is actually due, not to the peptone directly, but to its effect upon the liver. This old observation of the French investigators furnishes the exact counterpart of

the experiments already described in this paper on the production of incoagulability in anaphylaxis, and suggests the inference that peptone shock and anaphylactic shock resemble each other for the simple reason that both act upon the same mechanism.

As a matter of fact, poisons which have a marked effect upon the liver tend not only to render the blood incoagulable, but also to alter its chemical composition in almost exactly the same manner as does anaphylactic shock. Jobling (18) showed that anaphylaxis is accomplished by the following changes in the blood: (a) An increase in the proteolytic ferment; (b) a decrease in the antiferment; (c) an increase in the non-coagulable nitrogen; (d) an increase in amino-acids; (e) an increase in lipase.

The work of Opie, Barker and Dochez, and of Marshall and Rowntree, shows that in chloroform and in phosphorus poisoning the blood becomes distinctly less coagulable, and that it presents in addition every one of the features above described, with the exception of the decrease in antiferment. (This latter exception, in itself of little moment, is probably to be explained by the difference in technique.) Moreover, it seems to be fairly well established that the changes in the blood are roughly proportional to the degree of injury sustained by the liver. Indeed, it is stated by one of the authors previously mentioned that the proteolytic ferment present in the blood in these types of poisoning has characteristics which probably identify it with liver protease.

Thus we reach the conclusion that agencies which injuriously affect the liver tend to render the blood less coagulable, and to change its chemical composition and its biochemical properties in a characteristic manner. We know that anaphylaxis and peptones both render the blood incoagulable through their effect on the liver, and we are therefore entitled to believe that the chemical alterations so carefully analyzed by Jobling are probably due to the same mechanism. If this reasoning is correct, it ought to be possible to alter the typical course of anaphylactic shock by previous treatment of a sensitized animal with any one of the hepatic poisons above mentioned, for example, phosphorus or chloroform. There are serious difficulties, however, in the way

of securing clear cut evidence on this point. These poisons materially affect the general well being of the animal, while in most instances they only moderately lower the coagulation time. They do not, therefore, affect the hepatic mechanism of anaphylaxis to the same degree as do peptones, while, on the other hand, they produce other complicating changes. Nevertheless, it has been possible to show that phosphorus poisoning may to a certain extent modify anaphylactic shock. In the following experiment the dog died only after the lapse of several hours, instead of acutely in one hour, as do the sensitized controls, and with the symptoms of deferred instead of acute shock.

*Experiment.* Dog, weighing 12 pounds, sensitized by the intravenous injection of 5 cc. of horse serum. Two weeks later, coagulation time was found to be three minutes. Five cubic centimeters of phosphorized oil was now given by hypodermic injection. Two days later dog seemed sick and weak, and coagulation time was found to be sixteen minutes. The dog was tested at this point by the intravenous injection of 20 cc. of horse serum. Death did not occur within an hour, as typically in the sensitized controls, but only after an interval of more than six hours (during the following night). The immediate symptoms were also less violent.

Finally, the operation of injection into a portal branch, previously described for sensitized dogs, has been performed in normal animals with peptone as the injection material. The outcome has been identical with those observed in anaphylaxis, namely, a partial congestion of the liver, as shown by the following experiment.

*Experiment.* A normal dog weighing 13 pounds was etherized. The blood gave a coagulation time of four minutes. At 11.45, 2 cc. of a 10 per cent solution of Witte's peptone was injected into the left branch of the portal vein. The left lobe at once became darker in color, and swollen with blood; the right lobe remained normal in appearance. The blood was again tested at 11.50 and found to be incoagulable. At 12 the animal appeared to be in intense shock, and was therefore killed by opening the thorax. The liver was immediately removed from the body, and it was evident that the left lobe was con-

siderably more congested than the right, which, however, was also somewhat darker than before the injection.

The experiment presents a close parallel to those with the sensitized livers, not only in the localization of the congestion, but also in the rapidity with which a relatively minute dose, when injected directly into the portal vein, produces such pronounced effects as incoagulability of the blood and shock.

The foregoing facts justify the conclusion that peptone and anaphylactic shock affect the hepatic mechanism in an identical manner. Such being the case, the symptoms are necessarily alike. If one may make the assumption that the mechanism is to any degree exhaustible, it follows that a preceding injection of peptone will, for a short period, mitigate or abolish the effects of an injection of antigen into the sensitized animal. As a matter of fact this experiment had previously been performed with the above described result. Biedl and Kraus, however, who discovered the fact, have failed to interpret it in this light. They argue that since a previous injection of peptone protects a dog against a second injection of peptone within a short time, and, likewise, protects against anaphylactic shock, therefore, peptone must be the basis of anaphylactic shock. It is hardly necessary to point out the glaring fallacy of such logic. Morphine, given in sufficient amount, protects against the ordinary emetic effect of a small dose of the same drug, and, likewise, protects against the emetic effect of other drugs. One does not, however, argue that in the latter case morphine must be an intermediate product. The well known explanation of these facts is that morphine in large doses depresses the medullary center, and so excludes the emetic action of small doses of itself or of other drugs. In the same manner, peptone exhausts the liver both towards itself and towards other agencies acting upon the same mechanism.

#### VII. RELATIONS TO HUMAN SERUM SICKNESS

As far as I am aware, there have been no observations bearing upon the blood pressure or the coagulation time in serum sickness. As occasion has offered during the past year I have

made such observations, and have found that some of the cases present definite evidences of a departure from the normal. The blood pressure may not drop below 110 mm. of mercury. On the other hand, in the case of a perfectly healthy individual of about 30, who had received an injection of diphtheria antitoxin a week before, the systolic pressure fell to 90 mm. The coagulability of the blood is generally decreased when compared with the previous data secured in the same individual. The most marked delay was observed in a case which was examined for me at Bellevue Hospital by Dr. John Miller, in which coagulation was complete only after forty-eight minutes. These conditions are generally most marked at the time of onset of the serum sickness.<sup>2</sup>

It appears probable, therefore, that in human beings the hepatic mechanism plays some rôle. The effects are naturally less pronounced than in acute shock in the dog, but are still unmistakable in character. Acute shock in human beings might, of course, present more striking changes, but no opportunity has occurred to study such a case.

There is evidence (Friedberger (19)) also that the hepatic mechanism is involved in the anaphylaxis of guinea-pigs, resulting in an altered coagulability of the blood, but it does not appear to be a factor in the fatal outcome in these animals. The same is true of rabbits. Moreover, in both species shock is accompanied by congestion of the liver, but further study is required as to whether this effect is primary or secondary.

#### VIII. DISCUSSION

The evidence now available permits of the conclusion that anaphylactic shock in dogs results from the reaction of the sensitized liver to the antigenic substance. This conclusion is based, first, upon the experimental analysis of the individual symptoms; secondly, upon direct demonstration of the reactivity of the sensitized liver; thirdly, upon the pathology.

<sup>2</sup> After this had gone to press, appeared a paper by Shattuck, Arch. Int. Med., 1917, 20, 167, which largely confirms my own findings.



There are two characteristic manifestations of anaphylactic shock in the dog, namely, the incoagulability of the blood, and the fall in blood pressure accompanied by intra-abdominal congestion; to these might be added a third, chiefly, however, on account of its prominence in the literature, namely, the evidence of proteolysis in the blood. It has been demonstrated that the incoagulability of the blood is due directly to the action of the liver in anaphylaxis. Furthermore, it has been shown that the congestion of the liver is the immediate outcome of the local reaction, and that the general vasomotor mechanism is in no way concerned in its production. The fall in general blood pressure which accompanies anaphylactic shock in the dog is therefore interpreted as the secondary result of the hepatic congestion; the animal is, so to speak, bled to death into its own liver. If shock is prolonged and death deferred for some hours, the hepatic congestion may lead secondarily to congestion of the entire gastro-intestinal tract; this condition, however, is not in evidence if death occurs within an hour. Finally the chemical changes in the blood are such as are seen in other forms of hepatic injury, as in poisoning by chloroform or phosphorus, and are therefore in all probability the expression of the hepatic involvement in anaphylaxis.

The reactivity of the sensitized liver is demonstrated by the immediate congestive response to subcapsular injections of the antigen. If the antigen is injected into a branch of the portal vein, there is a corresponding lobar congestion. The normal liver shows no such changes.

The pathological anatomy of shock supports the view that the liver is the seat of the reaction. It is practically the only organ showing pronounced and gross alteration when the dog dies acutely. When death is deferred a series of secondary changes make their appearance. The microscopic study of the liver reveals severe congestion, and degenerative changes in the cells.

The blood of anaphylactic dogs is absolutely non-toxic for normal dogs, even in massive transfusion. This fact can only be interpreted to mean that the reaction of the liver does not liberate toxic substances into the general circulation. The

general effects do not, as a matter of fact, require any such explanation, and are adequately accounted for upon the basis of the local response of the liver. The proteolysis which Jobling demonstrated in the blood is not an evidence of toxicity, but is simply an innocent by-product of the hepatic reaction, just as is the incoagulability of the blood.

The preceding conclusions exclude the peptone theory of anaphylactic shock, which assumes that the symptoms are due to the constitutional effects of peptone-like substances produced in the course of the reaction and discharged into the blood. The peptone theory is based partly upon the fact that the symptoms of hepatic shock are identical with those of anaphylaxis, and partly upon the fact that a peptone injection temporarily desensitizes a sensitized dog. These facts, however, seem to be explicable upon quite different grounds. The symptoms are alike for the reason that in both cases they are mediated by the same hepatic mechanism. Peptone renders the blood incoagulable through a direct effect on the liver, as does anaphylaxis. Peptone induces a marked congestion of the liver through its local action, as does anaphylaxis. Desensitization by a preliminary peptone injection is, therefore, probably attributable to a temporary exhaustion of the hepatic mechanism. This view is borne out by the analogous retardation of shock in animals suffering from still another form of hepatic injury, namely phosphorus poisoning.

Anaphylaxis in the dog is, then, to be regarded as a cellular reaction involving the hepatic parenchyma. In this respect it differs from anaphylaxis in the guinea-pig, in which the liver does not materially participate. On the other hand, the reduction of anaphylaxis in the dog to a cellular basis demonstrates the essential uniformity of the reaction in the two species. In the guinea-pig the smooth muscle cells constitute the focus of the reaction, as do the liver cells in the dog. In neither species is there any evidence that secondary toxic products are formed, or that any changes of vital consequence take place in the blood.

As regards the hepatic mechanism in human beings there is no definite evidence. In serum sickness, however, there may

be a diminution of the coagulability of the blood and a lowering of the blood pressure, which are, perhaps, to be interpreted as evidences of hepatic involvement. In the guinea-pig and rabbit also there are distinct indications that the liver participates in the shock.

#### IX. SUMMARY

##### *1. Experimental data*

1. Blood taken from dogs at the height of anaphylactic shock fails to produce any symptoms when injected, even in very large amounts, into normal animals.

2. The isolated liver of a sensitized dog does not alter the coagulability of blood with which it is perfused; if, however, the antigenic substance is added to the blood before it traverses the organ, it passes out with its coagulability either diminished or completely abolished. The normal liver exercises no such effect.

3. The injection of the antigenic substance, even in very small amount, into one branch of the portal vein of a sensitized dog produces an immediate severe congestion of the corresponding lobe of the liver. The blood rapidly becomes incoagulable, and symptoms of shock develop. Subsurface injection of the antigen into the sensitized liver produces a local focus of congestion.

4. The postmortem examination of dogs dying in acute anaphylactic shock, within one hour, reveals as the single outstanding feature the enormous congestion of the liver. The other abdominal viscera show either very little congestion or none. The marked congestion of the gastro-intestinal tract in dogs dying after an interval of several hours is a late phenomenon, probably secondary to the congestion of the liver. Microscopic examination shows intense congestion of the liver and degenerative changes in the parenchyma cells.

5. Peptone affects the liver in exactly the same fashion as does anaphylaxis. The isolated organ, when perfused thereby, renders the blood incoagulable. Injection into one branch of the portal vein induces a localized area of hepatic congestion.

6. Human beings with serum sickness not infrequently show a low blood pressure, and diminished coagulability of the blood. Guinea-pigs also give evidence of diminished coagulability, indicating an hepatic involvement in the anaphylactic response.

## *2. Theoretical conclusions*

The available evidence indicates that in dogs the blood contains no toxic substance during anaphylactic shock. All of the symptoms of the shock appear to be traceable to the direct effect of the antigen upon the sensitized liver. This has been demonstrated of the incoagulability of the blood, and of the congestion of the liver itself. The latter phenomenon appears adequate to produce the fall in the general blood pressure by removing a large percentage of the blood from circulation. The evidences of proteolytic change in the blood are exactly the same as those which accompany other forms of hepatic injury, such as phosphorus or chloroform poisoning.

The peptone theory of hepatic shock maintains that peptones are produced during the anaphylactic reaction, and that they are directly responsible for the symptoms. There are no experimental observations which establish this theory. It is based chiefly on the resemblances between peptone intoxication and anaphylactic shock, and these are adequately explained by the fact that in both conditions the same hepatic mechanism is affected. The peptone theory cannot be harmonized with the experimental data recorded in this paper.

Anaphylactic shock in dogs, therefore, appears to be a cellular reaction, the site of which is the hepatic parenchyma. Other tissues may participate, but at present there is nothing to indicate that this is the case. In the guinea-pig the chief site of the cellular reaction is the smooth muscle tissue. The fact that in both species the fundamental mechanism is a cellular reaction, even though different tissues are involved, furnishes the basis for a uniform theory of anaphylaxis. The data herewith advanced indicate that the humoral theory of anaphylaxis (which is intimately bound up with the conception of a circulating

toxic product, such as anaphylatoxin), is inapplicable to dogs, as previous studies have shown it to be inapplicable to guinea-pigs.

The fact that peptone affects the liver in very much the same manner as does a specific antigen to which the organ is sensitized may serve to explain the therapeutic effects observed after the injection of this substance in certain infections, such as typhoid fever.

These results introduce us to a new function of the liver, namely its participation in the immune reaction. This participation does not appear to be indirect, as had previously been believed, namely by virtue of the toxic degradation of the antigen. On the contrary, it is a direct and immediate reaction profoundly involving the functions and structure of the organ. The accompanying general symptoms appear to be merely accidental by-products of this reaction, just as asphyxia in the guinea-pig is an accidental result of the contraction of the smooth musculature. It is perhaps needless to add that the hepatic reaction does not occur during the course of the infectious diseases in the exaggerated form induced by the anaphylactic experiment; but that it plays a more subdued and continuous rôle can hardly be doubted, in view of the direct evidence afforded by the study of the blood in human serum sickness. Similar effects upon coagulability as determined in anaphylactic guinea-pigs lead to the belief that the liver is probably a constant and important factor in the immune reaction throughout the mammalia.

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# TISSUE TRANSPLANTATION AND ANAPHYLAXIS

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The results of various investigations in the field of anaphylaxis, especially those of Schultz, Dale, Weil and Manwaring, definitely point to the conclusion that in the process of sensitization and anaphylactic reaction the union of the antigen with various tissues, the elaboration of antibodies on the part of the cells to which the antigen has been anchored, and the additional union of circulating antigen with the sessile cell-antibodies, are, if not the only factors, at least very important ones. Under certain conditions these changes in the sensitized cells lead to functional reactions on the part of the cells that are responsible for the anaphylactic shock and other anaphylactic phenomena of a more or less acute character. But we know that under certain conditions there may be anaphylactic reactions that entail morphological changes. Arthus has shown that in a sensitized rabbit necrosis of the sensitized tissue may follow the subcutaneous injection of the antigen. We know that in serum sickness, swelling of the joints and morphological changes in the skin may occur. It is even probable that the morphological changes produced by the tubercle bacillus or its products are in certain cases dependent upon a state of sensitization of the tissues, and that the formation of tubercles may represent an anaphylactic response. Introduction of pieces of tumor in a sensitized or immunized animal causes an acceleration of the lymphocytic reaction.

Under those conditions it was thinkable that if we sensitized tissues to a foreign protein like horse serum and transplanted the sensitized tissue into other animals, the behavior of the trans-

planted tissue might be influenced through a previous sensitization of the host animal with the same antigen, or through a subsequent injection of the antigen into the sensitized animal, after it had received the tissues. The life or the proliferative power of the transplanted tissue might be altered as a result of the serum, or the sensitization might lead to variations in the power of the tissue to attract lymphocytes from the vessels of the host. We, therefore, carried out a series of experiments in which we sensitized guinea-pigs with horse serum and subsequently transplanted pieces of uterus, in some cases thyroid, and in a few cases, also, ovaries of the injected guinea-pig, into other guinea-pigs which had or had not previously received injection of horse serum. In some cases the guinea-pigs were reinjected after transplantation, in others only a first injection was given. In different experiments the doses of the injected serum and the periods at which the injections were administered varied somewhat. At certain times following the transplantation the pieces were taken out and examined microscopically. In two cases autotransplantation of the tissues took the place of the usual homoiotransplantation.

#### *Series 1*

*Experiment 1.* One cubic centimeter of horse serum was injected into a female guinea-pig weighing 340 grams. Sixteen days later a piece of the uterus of the injected guinea-pig was transplanted into each of four male guinea-pigs, which had not previously been injected. In one horn of the uterus of the injected female pregnancy was found. Six days after transplantation the four guinea-pigs were injected subcutaneously with 0.5 cc. of horse serum. Pieces were taken out five and thirteen days following the injection; i.e., eleven and nineteen days following the transplantation.

Simultaneously with the uterus of the previously injected guinea-pig some pieces of uterus of a control guinea-pig not previously injected were transplanted into the same four guinea-pigs and were taken out simultaneously with the sensitized pieces. Both sets of pieces behaved normally; the character of the transplanted tissue and the activity of lymphocytes were approximately the same in both pieces. The injection of horse serum had not produced any definite effect.



*Experiment 2.* One-half cubic centimeter of horse serum was injected into a female guinea-pig. At the same time three male guinea-pigs were injected with 0.1 cc. of horse serum. Fifteen days later a piece of uterus and one of the thyroid glands of the injected female were transplanted subcutaneously into the sensitized male guinea-pigs. Pieces of uterus of a control guinea-pig not previously injected were transplanted into the other side of the male guinea-pigs. All the pieces were taken out five days after transplantation.

In two animals the pieces of uterus of the sensitized guinea-pig were infected, while the control pieces were normal. In one guinea-pig some surface epithelium, gland and connective tissue of the sensitized uterus were preserved, but in the control uterus considerably more tissue was preserved. The transplanted thyroid of sensitized guinea-pig, behaved like normal pieces of thyroid after transplantation into a normal animal.

*Experiment 3.* The uterus, ovaries and thyroids of a guinea-pig that had received a subcutaneous injection of 2 cc. of horse serum two days previously, were transplanted into male guinea-pigs that had received 0.1 cc. of horse serum fourteen days preceding the injection. Two of these guinea-pigs were reinjected intraperitoneally with 0.8 cc. of horse serum three days after the transplantation (seventeen days after first injection). One guinea-pig was not reinjected.

The pieces from the reinjected guinea-pigs were taken out five days (1-b) and twelve days (2-b) after the transplantation respectively; the pieces from the non-reinjected guinea-pig (4-b) were taken out twelve days after transplantation.

Piece 1-b shows some infection. Where leucocytes have not destroyed the tissue, surface epithelium, some glands and unstriated muscle are present. Some mitoses are found in the glands. In the thyroid the peripheral acini are preserved.

Piece 2-b. Uterus. Only connective tissue and perhaps endothelium are seen. Ovary. Germ epithelium, tunica albuginea, fat tissue and some connective tissue are preserved. A mitosis is seen under the germ epithelium. Some lymphocytic infiltration is present in the connective tissue and ovary near the fat tissue and also at some places in the germ epithelium.

Piece 4-b. Surface epithelium, glands, muscle and peritoneum are well preserved; gland and surface epithelium are of high cylindrical cells showing mitoses. The periglandular connective tissue is partly hyaline, partly somewhat myxoid. There is much lymphocytic

infiltration around the glands, and also in the connective and muscle tissue, but especially in the glands. Also the mucosa below the surface epithelium is infiltrated with lymphocytes. In this case the transplanted tissue was in a much better condition in the not reinjected animal.

*Experiment 4.* Injected one female and three male guinea-pigs, each with 0.05 cc. of horse serum. Six days later the female became ill. The uterus was transplanted into the three injected males. One horn of the uterus showed a pregnancy of hazelnut size. Nine days after the transplantation, i.e., fifteen days after the first injection male 2-c was injected with 0.3 cc. of serum and 3-c with 0.7 cc. serum; 4-c was not injected. All the pieces were taken out fifteen days after transplantation; i.e., six days after the second injection.

Piece 2-c. Surface epithelium, glands and connective tissue of the mucosa are preserved. Some mitoses are present in the glands. Some lymphocytes are seen under the epithelium. Most of the tissue is necrotic, and the necrotic tissue contains some polynuclear leucocytes. At places there is some lymphocytic infiltration. It is doubtful whether any unstriated muscle was preserved; if so, it is muscle which is infiltrated with lymphocytes.

Piece 3-c shows high cylindrical surface epithelium and glands with mitoses and a very cellular myxoid connective tissue with mitoses (almost predeciduomatous tissue) in some parts of the mucosa, in others hyaline connective tissue. Some unstriated muscle and peritoneal endothelium are preserved. There is slight lymphocytic infiltration in the surrounding connective tissue.

Piece 4-c is similar to 3-c. The same high cylindrical surface and gland epithelium with mitosis is present. The greater part of the unstriated muscle is necrotic, only a part of it being well preserved. The mucosa is cellular. A few lymphocytes have migrated through the glands. Some of the unstriated muscle is infiltrated with lymphocytes. In this case there is no effect of sensitization and of reinjection. The sensitizing doses had been small. The second injection was administered and the pieces were taken out at a relatively late period following transplantation.

*Experiment 5.* Female guinea-pig (310 grams) was injected with 0.1 cc. of horse serum. Twelve days later the uterus of the injected guinea-pig was transplanted into four guinea-pigs (1-d, 2-d, 3-d, 4-d) which had not been previously injected, and into a fifth (male) guinea-pig which had received 0.1 cc. of horse serum twenty-five days pre-

viously (5-d). Three embryos were found in the female guinea-pig, the uterus of which was used; the tissue above the place of pregnancy was transplanted. Six days after the transplantation guinea-pigs 1-d, 2-d, 3-d, 4-d were injected each with 1 cc. horse serum.

Piece 1-d (taken out nine days after transplantation, three days after injection). Uterine epithelium and glands are preserved, both with mitoses. Also unstriated muscle and peritoneal vessels and endothelium are preserved; there are mitoses in the fibroblasts.

Piece 2-d (taken out eleven days after transplantation, five days after injection). Some high cylindrical epithelium, and some glands are preserved. It is doubtful how much unstriated muscle has been preserved. Some lymphocytes have infiltrated the cellular and fibrous connective tissue. Much necrotic hemorrhagic material is present.

Piece 3-d (taken out fourteen days after transplantation of uterus, eight days after injection of horse serum). Surface epithelium is preserved. Where the connective tissue is cellular under epithelium, the latter is high cylindrical and contains mitoses; above the necrotic tissue it is vacuolar. Some glands are preserved. Some cellular (predeciduous) connective tissue is present around some glands. Some rarified unstriated muscle is preserved. There are some lymphocytes in the edematous connective tissue under the epithelium.

Piece 4-d (taken out eighteen days after transplantation, twelve days after injection). The peritoneal tissue is preserved.

Piece 5-d (taken out seven days after transplantation). The tissues in general are well preserved. Epithelium and glands are preserved. There is some hyaline connective tissue under the epithelium; at other places there is some cellular (transplanted) connective tissue around the glands. The transplanted muscle and peritoneal tissue are still alive. Some connective tissue is growing between the bundles of muscle tissue. There is no noticeable effect of the injections of horse serum on the transplanted tissue.

*Experiment 6.* A female guinea-pig weighing 490 grams received an injection of 0.5 cc. of horse serum. Twelve days later two male guinea-pigs were injected with 0.1 cc. of horse serum subcutaneously and with 0.1 cc. intraperitoneally. Twenty-five days following the injection of the female, and thirteen days following the injection of the male guinea-pigs, the uterus, thyroid and ovary were transplanted from the female into the male guinea-pigs. The transplanted uterus and ovary were taken out from male k-1 three days after transplantation and the transplanted uterus and thyroid were taken out from male k-3 twelve days after transplantation.

k-1. Uterus. The surface epithelium and a few small glands with some connective tissue below them are preserved; also parts of the peritoneal endothelium are preserved. In the ovary the germ epithelium and a zone of connective tissue below it with some small peripheral primordial follicles and some medullary tubules are preserved; the rest is necrotic.

k-3. The usual uterine tissues are well preserved. There are mitoses in the surface epithelium and in the connective tissue cells in the mucosa. Some lymphocytes have migrated through the surface and gland epithelium. Much of the unstriated muscle is necrotic.

There was, therefore, no effect of sensitization on uterus or ovary. The transplanted thyroid in k-3 showed only few acini. There was an absence of lymphocytic infiltration and of connective tissue ingrowth. This condition of the thyroid at this period after transplantation is not usual.

*Experiment 7.* Male guinea-pigs were injected each with 0.1 cc. of horse serum subcutaneously and with 0.1 cc. intraperitoneally. On the same day a female guinea-pig was injected with 0.5 cc. of horse serum. Fourteen days later the uterus, thyroid and ovary from the female were transplanted into males L-1, L-2, L-3. The left horn of uterus was found pregnant; the right horn was used for transplantation. Two days after the transplantation; i.e. sixteen days after the first injection, L-1 and L-2 were injected with 0.8 cc. of horse serum; L-3 did not receive a second injection.

L-1 and L-3 were taken out five days after the transplantation; i.e., nineteen days after the first injection or three days after the second injection.

L-2 was taken out twelve days after transplantation, ten days after second injection of L-2.

L-1. Uterus. A layer of fibroblasts surrounds the well preserved uterine surface epithelium and glands. At some places underneath the epithelium the connective tissue is much rarified, at other places it is hemorrhagically infiltrated. In the surrounding connective tissue are embedded some uterine epithelial structures with mitoses.

L-3 (without second injection). Only ovary is found. The germ epithelium and sub-epithelial connective tissue and fat tissue are well preserved, also the fimbria of the Fallopian tube is well preserved. Some primordial follicles with ova are preserved in the subepithelial connective tissue. The germ epithelium sends processes into the underlying connective tissue. Some mitoses are found in the germ epithelium.

L-2 (reinjecting). The transplanted uterus is well preserved with mitoses in the epithelial structures. The deeper parts are necrotic as usual. Some lymphocytes are present in the myxoid mucosa. In the thyroid the peripheral acini are preserved with some mitoses. There is some lymphocytic infiltration around some of the acini. The necrotic center is only incompletely organized with hyaline material.

No effect of the injections of horse serum was noticeable in the experiment.

*Experiment 8.* Guinea-pigs 2-g, 3-g, 4-g were injected with a little over 0.1 cc. horse serum subcutaneously. Twelve days later a female guinea-pig (360 grams) was injected with 2 cc. horse serum (1 cc. subcutaneously, 1 cc. intraperitoneally). Three days after the injection of the female and fifteen days after the injection of 2-g, 3-g, 4-g, the uterus, thyroid and ovary were transplanted into 2-g, 3-g, 4-g. Three days after transplantation 3-g was injected with 1 cc. horse serum intraperitoneally.

2-g (taken out five days after transplantation; i.e., twenty days after the injection). Some epithelium, glands, unstriated muscle and peritoneum are preserved. Some mitoses are seen in the surface epithelium and glands. Most of the tissue is necrotic.

3-g (taken out thirteen days after transplantation; i.e., twenty-eight days after first injection or ten days after second injection). The piece is necrotic; the epithelium and glands are shrunken. There is some lymphocytic infiltration near the epithelium. New connective tissue is organizing it. Thyroid: only fat tissue found.

4-g (pieces taken out thirteen days after the transplantation, i.e., twenty-eight days after the first injection). The uterus shows preserved low cuboidal epithelium with some mitoses; the transplanted fat tissue is well preserved; most of the tissue under the epithelium is necrotic. Some lymphocytes are present around the fat tissue. *Ovary:* The germ epithelium and some connective tissue below it are alive; the follicles are dead. Some lymphocytes are migrating through the germ epithelium.

In this case the reinjection of 3-g may possibly have been responsible for the difference in the tissue transplanted into 3-g and into 4-g.

*Experiment 9.* Four male guinea-pigs were injected with 0.1 cc. of horse serum intraperitoneally or subcutaneously and one female guinea-pig was injected with 0.5 cc. of horse serum. Fifteen days later the female guinea-pig was injected a second time with 1 cc. of horse serum. Thirty minutes after injection animal was weak; seventy-five

minutes later it began slowly to recover. On the following day (one day after first injection) the animal was well; its uterus and thyroid were transplanted into three of the injected males.

Male 2-h (found dead two days after transplantation; i.e., eighteen days after the injection of the male). The uterus is mostly necrotic; the superficial parts had been alive before postmortem changes had set in.

Male 3-h (pieces taken out twelve days after transplantation; i.e., twenty-eight days after the first injection). The uterus is well preserved, with some mitoses in the surface epithelium; some predeciduous connective tissue with mitoses is present under epithelium. At other places there is hyaline connective tissue. Some dilated glands and also some unstriated muscle are preserved. In the surrounding connective tissue are some masses of leucocytes. Also under the epithelium there is some lymphocytic infiltration.

Transplanted thyroid. The center is well organized. In the periphery the well preserved acini are surrounded by large masses of lymphocytes. Lymphocytes have destroyed some acini. Toward the center solid epithelial strands also are attacked by lymphocytes. In the third male the transplanted uterus had been infected.

No special effect of the horse serum injections was noticeable in this experiment.

*Experiment 10.* One female guinea-pig was injected with 0.5 cc. of horse serum. Twelve days later a piece of uterus was autotransplanted into the subcutaneous tissue. Three days after the transplantation; i.e., fifteen days after the first injection, 1 cc. of horse serum was intraperitoneally injected. Animal was weak after the injection. The uterus was taken out seven days after the transplantation; i.e., four days after the second or nineteen days after the first injection. The surface epithelium was found to be well preserved; high cylindrical cells and glands showing mitoses were present; fibrillar mucosa and unstriated muscle were partly preserved; there was much hemorrhagic-necrotic material. No definite effect of the horse serum injections was noticeable notwithstanding the weakness of the animal following the second injection. In this case autotransplantation of the uterus had been carried out.

*Experiment 11.* One male guinea-pig was injected with 0.2 cc. of horse serum subcutaneously. Twelve days after the injection its own thyroid and a piece of skin were autotransplanted subcutaneously. Fifteen days after the first injection; i.e., three days after the trans-

plantation 1 cc. of horse serum was injected intraperitoneally into the same guinea-pig. Pieces were taken out ten days after the transplantation; i.e., twenty-two days after the first or seven days after the second injection. The transplanted thyroid was well preserved in the periphery. There were frequent mitoses in the acini. In the center was loose connective tissue with some large vessels. Relatively little connective tissue was growing between acini. The transplanted skin was well preserved; there were many mitoses in the hypertrophic epithelium. No effect of horse serum injection on the autotransplanted tissue was noticeable.

*Experiment 12.* Female guinea-pig was injected intraperitoneally with 0.4 cc. of horse serum. Nine days later the uterus of the injected guinea-pig was autotransplanted subcutaneously. Four days after the transplantation; i.e., thirteen days after first injection, 1 cc. of horse serum was injected. Piece was taken out twelve days after transplantation, eight days after second, twenty-one days after first injection. Transplanted uterus well preserved. High cylindrical surface epithelium with some mitoses. Some glands with cylindrical epithelium and with mitoses. Underneath the epithelium was cellular connective tissue and at some places myxoid connective tissue with mitoses. Further away from the epithelium, underneath the subepithelial connective tissue, was some hyaline fibrous tissue without lymphocytes, traversed by capillaries and with some islands of well preserved connective tissue and some preserved unstriated muscle. There were some mitoses (regenerative in character) in the fibroblasts under the epithelium near the margin. There were a few lymphocytes in the epithelium. The autotransplanted uterus had not been affected through the injections of horse serum.

This first series of experiments showed that on the whole sensitization of tissues through injection of horse serum and subsequent transplantation of these sensitized tissues into previously sensitized guinea-pigs or into guinea-pigs that had not been previously sensitized, but were subsequently, i.e., following the transplantation, injected with horse serum, did not influence in any noticeable way the life or growth of the transplanted tissues nor the reaction of the host toward the transplants.

The outcome of experiments 3 and 8 suggested, however, the possibility that transplantation of sensitized tissues into sen-

sitized guinea-pigs, followed by a second injection of horse serum, may influence the condition of the transplanted tissue provided the uterus had been transplanted at an early date after injection of horse serum into the female. We therefore carried out a second series of four experiments in order to test the validity of this suggestion. For the sake of brevity we shall state the results of this series as a whole without differentiating between the individual experiments.

### *Series II*

Twelve guinea-pigs (mostly males) ranging in weight from 335 to 450 grams were injected subcutaneously with 0.15 cc. of horse serum (Lot I) twelve days later three female guinea-pigs weighing slightly over 400 grams were injected each with 1 cc. of horse serum subcutaneously and 1 cc. intraperitoneally. A fourth female of the same strain was not injected (Lot II).

*Lot II.* Two, three and four days after the injection of the females; i.e., fourteen to seventeen days after the injection of Lot I, the uterus and in some cases the thyroid of the injected females of Lot II were transplanted subcutaneously into the sensitized guinea-pigs of Lot I ( $A_1$ ,  $A_2$ ,  $A_3$ - $B_1$ ,  $B_2$ ,  $B_3$ - $C_1$ ,  $C_2$ ,  $C_3$ ). Pieces from the same female guinea-pigs were also transplanted into two not previously injected male guinea-pigs, which served as controls ( $A_4$ ,  $B_4$ ). All of these guinea-pigs with the exception of  $C_1$  and  $C_3$  received a subcutaneous injection of 1 cc. of horse serum two to three days after the transplantation of the pieces.  $A_1$ ,  $A_2$ ,  $A_3$ ,  $B_1$ ,  $B_2$ ,  $B_3$ , and  $C_2$  received therefore in addition to the sensitized pieces of uterus or thyroid, two injections of horse serum, one preceding and one following the transplantation.  $C_1$  and  $C_3$  received only one injection; namely, the one preceding the transplantation, and  $A_4$  and  $B_4$  received only one injection; namely, the one following the transplantation. In guinea-pigs  $D_1$ ,  $D_2$ ,  $D_3$ , which had received 0.15 cc. of horse serum seventeen days previously, and in control guinea-pig  $D_4$ , which had not been injected previously, pieces of uterus and thyroid from the fourth injected female guinea-pig of Lot II serving as control, were transplanted. Each of these four guinea-pigs received a subcutaneous injection of 1 cc. of horse serum two days after the transplantation.  $D_1$ ,  $D_2$ ,  $D_3$  received two, and  $D_4$  one injection of horse serum. All of the animals stood the injection of horse serum and transplantation of pieces well with the exception of



A<sub>2</sub>, A<sub>3</sub>, and B<sub>2</sub>. These latter animals were weak and quiet after the second injection. The pieces were taken out from A<sub>2</sub> six days, and from B<sub>2</sub> five days after the transplantation. The pieces of all the other animals were removed ten or eleven days after transplantation.

The results of the microscopic examination were as follows:

In all the guinea-pigs with exception of A<sub>2</sub>, A<sub>3</sub> and B<sub>2</sub>, the pieces of uterus and thyroid were on the whole normal. The surface epithelium formed a more or less complete cyst; it was often high cylindrical and usually contained mitoses. The glands also frequently had high cylindrical epithelium and showed mitoses often. At other places they were well preserved and without mitoses. Mitoses were not found in the glands of all the guinea-pigs. The condition of the glands depended in part upon the character of the surrounding connective tissue; if the latter was well preserved and cellular, the glands were usually in a good state; if the connective tissue was hyaline, the glands were usually vacuolar and without mitoses. The preservation of unstriated muscle and peritoneal endothelium varied in different pieces. But there was evidently no regularity in the variations. There was no direct connection between the condition of the transplanted uterus and thyroid and the injections of horse serum. There was no noticeable difference between the pieces in guinea-pigs which had been injected once or twice. The previous injection with horse serum of the guinea-pig, the uterus and thyroid of which were used for transplantation, did not markedly affect the results. There was, however, more marked lymphocytic infiltration of the transplanted thyroid than of the uterus. One guinea-pig, which had received one injection of horse serum (C<sub>1</sub>) and another guinea-pig, which had received two injections of horse serum (D<sub>2</sub>) were found in an early period of pregnancy, and the injections did not interfere with the progress of the pregnancy. In another guinea-pig (B<sub>3</sub>) ovulation had taken place about seven days following the second injection of horse serum. The uterus transplanted in guinea pigs B<sub>3</sub> and C<sub>1</sub> was, however, perhaps not quite so well preserved as in the other guinea-pigs of the same series.

If we now consider separately the pieces transplanted in guinea-pigs A<sub>2</sub>, A<sub>3</sub> and B<sub>2</sub>, which animals were more markedly affected by the second injection of horse serum, we find the following results:

A<sub>2</sub>. The piece of uterus was taken out six days after transplantation; i.e., three days after the second injection. The uterus was found to be free in the host, without junction with the surrounding host tissue. Most of it was necrotic, but a surface area to which the plasmatic fluids

of the host had access (epithelium, glands, connective tissue of mucosa, also some unstriated muscle under the peritoneum), were preserved. A mitosis was seen in the surface epithelium.

A<sub>3</sub> (taken out ten days after transplantation; i.e., seven days after second injection). This piece was also loose, without connection with the surrounding host tissue. Almost the whole piece was necrotic, but some epithelium and some connective tissue below, and a few blood vessels under the epithelium were alive. The thyroid was necrotic.

B<sub>2</sub> (taken out five days after transplantation). The transplanted uterus was almost all necrotic and shrunken. At one place a few living glands, surrounded by fibroblasts, were seen. Also some peritoneal endothelium and some unstriated muscle were preserved under the peritoneum. But on the whole very little tissue was preserved.

We see, therefore, that an effect of the injections was apparently only visible in three animals, the general condition of which had been much affected as the result of the second injections. This suggests that the general circulatory, metabolic consequences of the second injection may influence the fate of the transplanted tissue. It would, however, be desirable to test in further experiments this suggestion which we can at present merely consider as provisional.

#### CONCLUSIONS

From these experiments we may draw the conclusion that if the uterus and thyroid of animals that had previously received injections of horse serum into guinea-pigs that had been injected with horse serum previous to or subsequent to the transplantation, or both previous and subsequent to the transplantation, the treatment with horse serum, does not have any distinct influence on the life and growth of the transplanted piece, neither does it noticeably alter the lymphocytic reaction on the part of the host tissue.

The outcome of our second series of experiments suggests however, that in cases in which the second injection of horse serum affects markedly the general state of health of the host animal, the transplanted pieces may suffer. This was found to be so in three animals (A<sub>2</sub>, A<sub>3</sub>, B<sub>2</sub>). In an experiment of the first series,

in which a second injection produced marked effects in an animal in which an autotransplantation of the uterus had been carried out, the condition of the transplanted uterus was not influenced in an unfavorable way.

The lack of effect of the injections in the majority of the experiments does not of course exclude the possibility that with still further variations in dosage or time relations an influence of the sensitization to horse serum may be demonstrable. It might be especially desirable to repeat the experiment choosing dosage and time of injection in such a way that the second injection has a definite effect on the general condition of the guinea-pig. If it should again be found that in those animals in which the second injection produces general effects, the state of preservation of the transplanted piece is interfered with, we would have to decide further whether in this case we are dealing with a specific effect of the injections on the transplanted piece or with a non specific effect, due to interference with the proper nourishment of the tissue as the result of circulatory and general metabolic changes in the host.



## STUDIES IN ANAPHYLAXIS

### XXII. ANAPHYLACTIC REACTIONS OF THE ISOLATED DOG'S LIVER

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This study, which was being conducted in connection with the work described in the preceding paper, has been interrupted by the war. In view of the indefinite postponement of its complete publication, it seems advisable at the present time to give a brief preliminary statement of the results, in so far as they have been clearly established. The following protocol of one of the experiments is illustrative of the essential findings.

The liver was removed from a dog actively sensitized to horse serum, and placed in a warm perfusion chamber. By means of a cannula tied in the portal vein the liver was washed out with a liter of salt solution. The defibrinated blood of the same animal was divided into equal portions of 200 cc. each. The organ was then perfused with one of these portions, the blood being passed through a number of times successively. This blood, after the final passage, was used to transfuse a small normal dog, after removing an approximately equal quantity of blood by the carotid. The animal manifested no change as the effect of this operation.

To the second portion of blood was added 5 cc. of horse serum and perfusion was again performed as before. Under these conditions the liver promptly underwent a striking change. The organ became intensely congested and swollen. Measurement of the perfusion fluid showed that a considerable amount was retained by the liver. After a number of perfusions, this blood also was used to transfuse a normal dog, which likewise manifested no abnormal effects.

These experiments confirm and extend the conclusions of the preceding paper. They show, in the first place, that no toxic substance is added to the blood when it carries the antigenic substance through a sensitized liver. In the second place, they show that the congestion of the liver which ensues under these circumstances is a purely local effect, produced in the organ itself, and in no manner dependent upon changes induced in the extra-hepatic sympathetic system.

The mechanism of the hepatic reaction has not been completely analyzed; the following facts, however, seem to throw light upon it. The normal liver presents no change when perfused by the antigen of these experiments (horse serum) mixed with defibrinated blood, whether the latter is derived from normal or sensitized dogs. The sensitized liver, on the other hand, shows the typical reaction when thus perfused, irrespective of the origin of the blood. Perfusion of the liver with the antigen in Locke's fluid appears to produce no effect. Thus, in addition to the sensitized liver and the antigen, blood seems to be essential, but apparently as a physiological, not an immunological factor, in the reaction.

Finally, it has been found that the isolated normal liver becomes engorged when perfused with peptone, thus confirming the view that the reaction of the liver to peptone is essentially identical with the anaphylactic reaction of that organ. /

## A NEW METHOD FOR MAKING WASSERMANN ANTIGENS FROM NORMAL HEART TISSUE

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One of the main difficulties in relation to the Wassermann reaction has always been the production of suitable antigens. Since Wassermann, Neisser and Bruck (1) first called attention to the possibility of employing the complement fixation test in the diagnosis of syphilis, the question of the best way of making and preserving antigen has come up from year to year. Wassermann started out by using extracts of livers or spleens of the congenitally syphilitic foetus, made by cutting up these organs into very small pieces and mixing this tissue with physiological salt solution to which phenol in the proportion of 0.5 per cent had been added. This mixture is shaken for twenty-four hours, then allowed to stand and the turbid supernatant solution, not the precipitate, is used as an antigen. From the very beginning Wassermann called attention to the fact that only such antigens are suitable which did not inhibit hemolysis with normal sera in double the quantity to be employed in the actual test.

Marie and Levaditi (2) found that just as satisfactory antigens could be made from normal, human and animal livers with the same method. Porges and Meier (3) after proving that the antigenic part of Wassermann's physiological salt extract was alcohol soluble, used alcoholic extracts of syphilitic livers, evaporated these extracts to dryness in vacuo and made emulsions of the sticky mass. They thought that the active principle of this residue was lecithin. They, as well as Levaditi and Yamanuchi (4), showed that cholesterin alone does not have any antigenic properties.

Landsteiner, Mueller and Poetzl (5) used alcoholic extracts of guinea-pig heart or liver and preserved these extracts at room temperature. This was a great step in advance, as such an antigen, being more stable, was usable for a longer period of time than the physiological salt extract, which besides had to be preserved in the refrigerator.

Thus gradually the fact became known that Wassermann antigens are lipoids, alcohol soluble substances. It was further shown that the antigenic strength of these alcoholic lipoidal solutions can be increased by the addition of cholesterolin.

It was Noguchi (6) who first definitely formulated a system of titration for judging the value of a given antigen. He clearly stated that the more one increases the minimal dose of an antigen necessary for obtaining a positive reaction with a known positive serum, the greater are the chances for picking up a doubtful reaction, and that in general at least five times the dose of antigen necessary to inhibit hemolysis with a positive serum should be used in performing the actual test. This dose must not inhibit hemolysis with negative sera, and must not produce hemolysis when incubated with corpuscles alone. Thus there are three essential facts which must be known about every antigen—its antigenic, its anticomplementary, and its hemolytic properties. In other words, what is the highest dilution in which an antigen will completely inhibit hemolysis with a known positive serum, what is the highest dilution in which it will still inhibit hemolysis with a negative serum, and what is the highest dilution in which it will still hemolyse red cells?

Noguchi (7) also classified the lipoids according to their solubility on the basis of the above mentioned titration. He thus produced his well known acetone insoluble antigen. This is nothing more than the ether soluble and acetone insoluble portion of the alcoholic extract of a normal heart, liver or kidney, a mixture of various phosphatides.

Today three kinds of antigen are in general use, the plain alcoholic extracts, the cholesterolinized plain extracts and the acetone insoluble preparations. Synthetic antigens all of which have lecithin as a basis have been used with more or less success by



Sachs and Rondoni, (8) Shuermann (9) and others. Any one of the three previously mentioned antigens is useful if properly titrated. In general the plain extracts are the weakest, the acetone insoluble preparations stronger, and the cholesterinized plain extracts the strongest of all.

Our laboratory uses all three of these alcoholic solutions adding 0.2 per cent cholesterin to a plain extract in order to produce the cholesterinized product. The prescribed properties for an antigen, that is usable, are as follows: it must not be hemolytic even in a dilution with physiological salt solution of 1 in 5. It must not be anticomplementary more than 1 in 10 in the beginning. It must bind or in other words inhibit hemolysis with a known positive serum at least in a dilution with a denominator that is greater by ninety than the denominator of the dilution in which it is anticomplementary. Thus if an antigen is anticomplementary in a dilution of 1 in 5, it must bind in a dilution of at least 1 in 95.

The Japanese have been using a dried fish called "Katsuwo-bushi" for antigen, making an alcoholic extract of the light yellow part of the flesh of this fish which belongs to the mackerel family. This dried fish is very hard. Through the kindness of Prof. Imamura of Kioto, we obtained a piece of the fish and produced a usable antigen of fish lipoids with the following properties: Not hemolytic in a dilution of 1 in 5 with physiological salt solution; not anticomplementary diluted 1 in 5; binds diluted 1 in 100. It is therefore evident that drying even for months under unfavorable conditions is not necessarily fatal to the antigenic value of the lipoids involved.

One great difficulty in making antigens has been to produce plain extracts and acetone insoluble preparations that were always usable, if prepared with uniform technique. This difficulty is not one confined to our laboratory, but is a difficulty that all serological workers have. One heart may make an excellent antigen while the next six give worthless extracts. It therefore seemed worth while to investigate the lipoids of the heart, to endeavor to classify them anew as to their antigenic value, to find out the reason for the variable results thus far ob-

tained, and to evolve a method of obtaining a good antigen every time.

With this end in view, the work of Erlandsen (10) was repeated and each one of his lipoidal fractions of beef heart was titrated for binding power and anticomplementary and hemolytic properties. The main and all-important procedures by which he achieves a separation of the lipoids from one another are, first, drying the ground beef heart muscle with the aid of a heated air current and later a vacuum desiccator and, secondly, the division of the extracts of this dried and pulverized tissue into a primary ether soluble portion and a secondary alcohol soluble portion. As to the chemistry of this procedure we quote Erlandsen:

It has been shown that the primary ether extract, which was neglected by almost all former investigators, and which later investigators mixed with the alcohol extract, not only contains large quantities of phosphatides, but also that the latter are different from those of the alcohol extract.

Erlandsen further states that if a primary alcohol extraction is made and this is followed up by a secondary ether extraction, substances are obtained, which are entirely different from those substances obtained by a primary ether extraction. Some phosphatides such as cuorin and cephalin, though insoluble in cold absolute alcohol when pure, are soluble in alcohol when mixed with other lipoids because they are soluble in the latter. Thus an alcoholic extract of wet beef heart gives a varying mixture of numerous phosphatides, while Erlandsen's method of the primary exhaustion of the dried tissue with ether and the secondary exhaustion of the same tissue with alcohol, allows one to separate them into two main groups.

The tables will best explain the antigenic value of the various groups. From Table A we see that the primary ether extract contains no substances that have any antigenic value except lecithin (C). This substance comprises less than one-quarter of the entire weight of the ether extract and though it has a fairly high binding power, it is anticomplementary 1 to 20. These

TABLE A

750 grams of dried pulverized beef heart

*Ether Extract*

Weight after evaporation under reduced pressure—66 grams

Dissolved in water free ether

Soluble portion

Precipitated with acetone

Insoluble portion

Grayish white substance  
(A)

Weight 3 grams

Saturated emulsion in 0.85  
per cent NaCl solution

Does not bind

Not anticomplementary

Not hemolytic

Soluble portion

Fats with slight amounts of  
phosphatides (B)

Weight 15 grams

Saturated solution in abso-  
lute alcohol

Binds 1 to 1600

Anticomplementary 1 to  
1600

Hemolytic 1 to 40

Insoluble portion

Redissolved in absolute  
ether

Soluble portion

Precipitated with absolute  
alcohol

Insoluble portion same as A

Weight 2 grams

Does not bind

Not anticomplementary

Not hemolytic

Soluble portion

Lecithin (C)

Weight 15 grams

Saturated solution in abso-  
lute alcohol

Binds 1 to 800

Anticomplementary 1 to 20

Not hemolytic

Insoluble portion

Treated with absolute alco-  
hol at 60° C.

Soluble portion

Cephalin (D)

Weight when purified 4  
gramsSaturated emulsion in 0.85  
per cent NaCl solution

Binds 1 to 20

Anticomplementary 1 to 20

Not hemolytic

Insoluble portion

Cuorin (E)

Weight when purified 3  
gramsSaturated emulsion in 0.85  
per cent NaCl solution

Binds 1 to 30

Anticomplementary 1 to 30

Not hemolytic

facts correspond with the findings of other investigators. Table A also shows that the ether extract contains large amounts of fats and fatty acids (*B*), substances which are very hemolytic in their properties. The other substances, organic salts (*A*), cephalin (*D*), and cuorin (*E*), can be considered neutral in their binding power, only increasing the anticomplementary titer of a mixture. Summing up we find that only one-quarter by weight of the ether soluble substances of dried pulverized beef heart have any antigenic value, and that three-quarters by weight of these substances are either very hemolytic or very anticomplementary.

The secondary alcohol extract gives us quite different results. Whereas a sample of the primary ether extract shows little binding power with a rather high hemolytic and a very high anticomplementary titer, the secondary alcohol extract shows high binding power with a low anticomplementary titer and no hemolytic properties. From the very beginning it was therefore evident that we could expect to find the active principle in this group of alcohol soluble substances.

The separation of the various fractions as shown in Table B, proved that our deductions were correct. By far the greater portion of the alcohol soluble substances falls into group (*E'*), which is composed of diaminomonophosphatides. The binding power of these lipoids is spectacular. We have repeated the separation five times and group *E'* has always shown the same antigenic power. Once a saturated solution in absolute alcohol of the diaminomonophosphatides bound in a dilution of 1 in 200,000. Groups *A'* *B'* *C'* may be classed as substances which interfere more or less with the antigenic value of *E'* when mixed with this group. Group *D'* comprises such a small part by weight of the entire quantity of alcohol soluble phosphatides that it may be neglected.

In making an antigen the more Group *E'* predominates in a given solution the better the antigen. It is clear that Noguchi's method excludes groups *B'* and *C'* (musclenucleon ?) and part of *A'* (inorganic salts) of table B, as well as group *B* (fats) of table A. However, it includes all the other groups of table A, which have no value as antigens with the exception of group

TABLE B

750 grams of dried pulverized beef heart

*Secondary Alcohol Extract*

Weight after evaporation under reduced pressure—70.5 grams

Dissolved in absolute alcohol

Soluble portion

Evaporated under reduced  
pressure  
Treated with water free  
ether

Soluble portion

Insoluble portion

Light yellow transparent  
mass (musclenucleon ?)  
(B')

Weight 16 grams

Saturated solution in 95 per  
cent alcohol

Binds 1 to 10

Anticomplementary 1 to 10

Hemolytic 1 to 10

Precipitated with acetone

Soluble portion

Substances like B' with  
slight amounts of phos-  
phatides (C')

Weight 7 grams

Saturated solution in 95 per  
cent alcohol

Binds 1 to 80

Anticomplementary 1 to 50

Hemolytic 1 to 10

Insoluble portion

Treated with absolute  
alcohol

Soluble portion

Diaminomonophosphatide  
(E')

Weight 37 grams

Saturated solution in abso-  
lute alcohol

Binds 1 to 12,800

Anticomplementary 1 to 10

Not hemolytic

Insoluble portion

Jecorin (D')

Weight 1.5 grams

Saturated emulsion in 0.85  
per cent NaCl solution

Binds 1 to 10

Anticomplementary 1 to 10

Not hemolytic

C (lecithin), since all substances of Noguchi's antigen are soluble in ether and insoluble in acetone. The slight advantage of thus adding lecithin to the diaminomonophosphatide group is far outweighed by the disadvantages of the anticomplementary titers of the other groups. Alcohol takes up just so much and no more of a mixture of phosphatides, whether this mixture consists essentially of alcohol soluble groups or of alcohol insoluble groups kept in solution by being themselves dissolved in alcohol soluble groups.

The average quantities by weight of the various groups are those shown in our tables,—whenever six to eight hearts are used. These averages vary within wide limits for each individual heart. It is therefore easy to understand that if a certain heart contains more than the average of primary ether soluble substances and less than the average of secondary alcohol soluble substances, this heart will not be likely to give us a good antigen if Noguchi's method is used. It will be quite useless indeed for making a plain alcoholic extract, since the facts that apply to Noguchi's method are still more important in the case of plain alcohol extracts, where no separation of the various phosphatide groups is attempted. Beside this the mechanical preparation of the tissue makes a difference. A finely ground heart will give off more of the secondary alcohol soluble substances than a coarsely ground one. The latter substances seem to be more firmly bound to the organized structure of the cell than the former. Erlandsen (10) has even referred to them as intracellular phosphatides as opposed to extracellular phosphatides, the primary ether soluble groups.

Considering all these facts and using the knowledge gained we propose the following methods for making antigens:

Take a normal beef heart and remove all the endo- and pericardium, larger blood vessels and fat. Grind the heart muscle very fine and spread it in a thin layer on glass plates. Dry by means of an electric fan for twenty-four hours, turning the layer of tissue after twelve hours. A parchment like dry sheet of tissue results. Break this up and grind again, thus producing a fine dry powder. Put this in bags and dry in an incubator at 37°C. for

several days. Extract with ether in a Soxhlet apparatus for twelve hours or in an ordinary wide mouthed bottle at room temperature five or six times until the supernatant ether is no longer colored yellow. The latter procedure takes from eight to ten days. The ether should cover the muscle powder to a depth of from 2 to 3 inches. Dry the powder by spreading it out on a sheet of paper for several hours. Now extract the powder with absolute or 95 per cent alcohol for ten days. The alcohol should cover the powder to a depth of half an inch and it will gradually assume a light yellow color. Titrate the antigen thus obtained. Extract the powder a second time with alcohol for fourteen days. Titrate this antigen.

Both extracts will prove to be good plain extracts, sometimes the second will be better than the first for the diaminomono-phosphatides seem to go into solution after groups *B'* and *C'* have been dissolved out.

In order to produce an acetone insoluble extract the plain extracts are united, evaporated to dryness at room temperature by means of a fan and treated with an excess of ether. The ether soluble portion is filtered off, allowed to evaporate, treated with an excess of acetone and the sticky brown residue is finally dissolved in a minimum quantity of absolute alcohol. The solution is then filtered and again titrated.

This preparation consists almost entirely of group *E'* and with ten hearts prepared in succession has each time given an antigen which bound at least 1 to 1600 and showed a low anti-complementary titer and no hemolytic properties.

A cholesterinized antigen can be made by adding 0.2 gram of cholesterin to 100 cc. of the plain extract or of the acetone insoluble preparation. The cholesterin will dissolve very readily in either of these phosphatide solutions, much more readily than in the same amount of absolute alcohol. After the addition of cholesterin the antigen is again titrated.

These antigens have been in use in our laboratory for the past six months. They have proven reliable in every way and have solved the problem of finding a certain and definite method of obtaining a reliable, fairly equal, and powerful product. Though

these antigens pick up weaker positives than our former preparations they have never given false positives.

A drying apparatus such as Erlandsen (10) described in his original article is not absolutely necessary for the preparation of the ground heart muscle. A vacuum desiccator for the secondary more thorough drying can also be dispensed with, without enough injury to the phosphatides to inhibit their antigenic value.

We have purposely refrained as much as possible from discussing the chemistry of the phosphatides. Many of the questions dealing with this group of substances cannot be considered definitely settled. We are aware of the fact that MacLean (11) has claimed that Erlandsen's diaminomonophosphatides are nothing more nor less than impure lecithin. This claim is not borne out by the biochemical behavior of lecithin compared to the diaminomonophosphatide group. A 2.5 per cent solution of purified lecithin in alcohol binds 1 to 1600, is anticomplementary 1 to 50 and is not hemolytic. A 2.5 per cent solution of purified diaminomonophosphatide in alcohol binds 1 to 25,000, is anticomplementary 1 to 5 and is not hemolytic. Both substances were made from the same dried heart tissue and treated according to MacLean's (11) method of purification. Furthermore our analytical results have thus far failed to verify MacLean's claim. However we wish to leave this issue open and to discuss this phase of the question as well as the question of the chemical changes which bring about a deterioration of the binding power of an antigen in a later paper.

#### SUMMARY

The antigenic value and anticomplementary and hemolytic properties of the various lipoids of beef heart have been investigated. It was found that lecithin is the only substance of the primary ether extract of dried pulverized beef heart that had any antigenic value, and that the most important substance as regards antigenic value occurs in the secondary alcohol extract and is a diaminomonophosphatide. On this basis of solubilities new methods for making antigens have been described.



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